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**Skin sensitization in silico protocol**

<http://researchonline.ljmu.ac.uk/id/eprint/13223/>

### Article

**Citation** (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

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# Journal Pre-proof

Skin sensitization *in silico* protocol

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PII: S0273-2300(20)30114-8

DOI: <https://doi.org/10.1016/j.yrtph.2020.104688>

Reference: YRTPH 104688

To appear in: *Regulatory Toxicology and Pharmacology*

Received Date: 24 February 2020

Revised Date: 18 May 2020

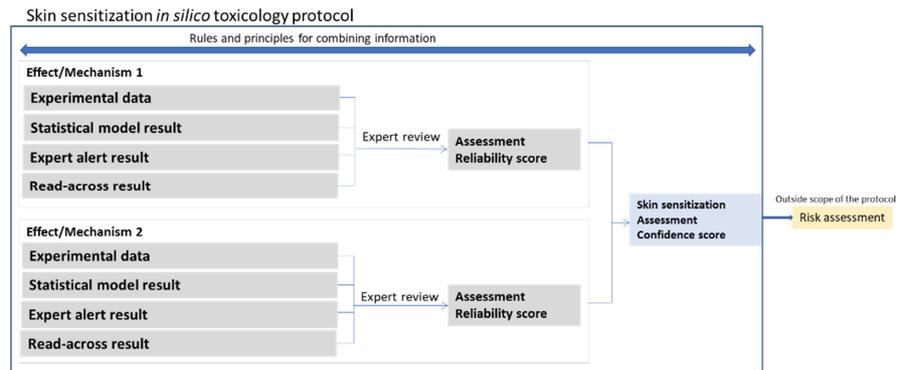
Accepted Date: 21 May 2020

Please cite this article as: Johnson, C., Ahlberg, E., Anger, L.T., Beilke, L., Benigni, R., Bercu, J., Bobst, S., Bower, D., Brigo, A., Campbell, S., Cronin, M.T.D., Crooks, I., Cross, K.P., Doktorova, T., Exner, T., Faulkner, D., Fearon, I.M., Fehr, M., Gad, S.C., Gervais, V., Giddings, A., Glowienke, S., Hardy, B., Hasselgren, C., Hillegass, J., Jolly, R., Krupp, E., Lomnitski, L., Magby, J., Mestres, J., Milchak, L., Miller, S., Muster, W., Neilson, L., Parakhia, R., Parenty, A., Parris, P., Paulino, A., Paulino, A.T., Roberts, D.W., Schlecker, H., Stidl, R., Suarez-Rodriguez, D., Szabo, D.T., Tice, R.R., Urbisch, D., Vuorinen, A., Wall, B., Weiler, T., White, A.T., Whritenour, J., Wichard, J., Woolley, D., Zwickl, C., Myatt, G.J., Skin sensitization *in silico* protocol, *Regulatory Toxicology and Pharmacology* (2020), doi: <https://doi.org/10.1016/j.yrtph.2020.104688>.

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## Graphical abstract



**1 Skin sensitization *in silico* protocol**

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63 **Glossary of acronyms**

Ac	Acylation
ACD	Allergic Contact Dermatitis
ADRA	Amino Acid Derivative Reactivity Assay
AOP	Adverse Outcome Pathway
ARE	Antioxidant/electrophile response element
BT	Buehler test
CD54	Cluster of Differentiation 54, a co-stimulatory adhesion molecule that is expressed in dendritic cells
CD86	Cluster of Differentiation 86, a co-stimulatory adhesion molecule that is expressed in dendritic cells
CV <sub>70</sub>	Concentration of test chemical yielding a cell viability of 70% in the U-SENS™ method
DA	Defined Approach
DC	Dendritic cells
DIP	Data interpretation procedure
DPRA	Direct Peptide Reactivity Assay
DSA <sub>05</sub>	Dose per skin area that produced a positive response in 5% of the tested population
EC <sub>1.5</sub>	Lowest concentration inducing a 1.5-fold change in luciferase activity in the assays measuring KE2
EC <sub>150</sub>	Effective concentrations yielding a relative fluorescence intensity [RFI] of 150% for CD86 in the h-CLAT test
EC <sub>200</sub>	Effective concentrations yielding a relative fluorescence intensity [RFI] of 200% for CD54 in the h-CLAT test
EC3	Effective concentration of a test chemical that gives a stimulation index with a three-fold increase over the vehicle control in the LLNA
EC <sub>3</sub>	Concentration with 3 fold luciferase induction in the KeratinoSens™ test
GARD	Genomic allergen rapid detection
GPMT	Guinea Pig Maximization test
GST	Glutathione S-transferase
HAF	Hazard assessment framework
h-CLAT	Human Cell Line Activation test
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
hTCPA	human T cell priming assay
IATA	Integrated approach to testing and assessment
IC	Induction concentration in GPMT
IC <sub>50</sub>	Concentration for 50% reduction of viability in KeratinoSens™ test
IL-18	Interleukin-18
IL-8	Interleukin-8
IL-8 Luc	Interleukin-8 Reporter Gene Assay
KE	Key Event
KE1	Key event 1: Covalent interaction with skin proteins
KE2	Key event 2: Events in keratinocytes

KE3	Key event 3: Events in dendritic cells
KE4	Key event 4: Events in lymphocytes
Keap1	Kelch-like ECH-associated protein 1
LLNA	Local Lymph Node Assay
LOEL	Lowest observed effect level
Log K <sub>ow</sub>	n-octanol/water partition coefficient
MA	Michael addition
MHC	Major histo-compatibility complex
MIE	Molecular initiating event
NOEL	No Observed Effect Level
NQO1	NADPH-quinone oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OECD	Organization for Economic Co-operation and Development
QMM	Quantitative Mechanistic Models
(Q)SAR	(Quantitative) Structure-Activity Relationship
RFI	Relative fluorescence intensity
SB	Schiff base formation
SI	Stimulation index
SLS	Sodium lauryl sulfate
SM	Supplementary material
SN1	Unimolecular nucleophilic substitution
SN2	Bimolecular nucleophilic substitution
SNAr	Nucleophilic aromatic substitution
STS	Sequential Testing Strategy
U-SENS™	U937 cell line activation Test

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74 **Abstract**

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76 The assessment of skin sensitization has evolved over the past few years to include *in vitro* assessments  
77 of key events along the adverse outcome pathway and opportunistically capitalize on the strengths of *in*  
78 *silico* methods to support a weight of evidence assessment without conducting a test in animals. While  
79 *in silico* methods vary greatly in their purpose and format; there is a need to standardize the underlying  
80 principles on which such models are developed and to make transparent the implications for the  
81 uncertainty in the overall assessment. In this contribution, the relationship of skin sensitization relevant  
82 effects, mechanisms, and endpoints are built into a hazard assessment framework. Based on the  
83 relevance of the mechanisms and effects as well as the strengths and limitations of the experimental  
84 systems used to identify them, rules and principles are defined for deriving skin sensitization *in silico*  
85 assessments. Further, the assignments of reliability and confidence scores that reflect the overall  
86 strength of the assessment are discussed. This skin sensitization protocol supports the implementation  
87 and acceptance of *in silico* approaches for the prediction of skin sensitization.

88 **Keywords:** *In silico*, *in silico* toxicology, computational toxicology, computational toxicology protocols,  
89 (Q)SAR, expert alerts, expert review, skin sensitization, defined approach, integrated approaches to  
90 testing and assessment (IATA), extractables and leachables.

91

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## 151 **1. Introduction**

152 Allergic contact dermatitis (ACD) is a common skin condition that results from the induction of a dermal  
153 immunological response after repeated exposure to a skin-sensitizing substance. ACD poses a significant  
154 public and occupational health concern, and much effort has been dedicated to the identification and  
155 classification of skin sensitizers. Historically, assessors have relied on human (Human repeat insult patch  
156 tests (HRIPT) and Human maximization tests (HMT)) or animal testing, the latter commonly using guinea  
157 pig (Guinea pig maximization (GPMT) and Buehler tests(BT))(Organisation for Economic Co-operation  
158 and Development (OECD), 1992) and mouse models (Local lymph node assay (LLNA))(OECD 2010a) to  
159 identify potential skin sensitizers. The guiding principles of the “3Rs” (replacement, reduction, and  
160 refinement) as applied to animal research(RUSSELL and BURCH 1959) have influenced the  
161 implementation of regulations, such as the 7th amendment of the Cosmetic Directive (Council Directive  
162 76/768/EEC of 1976-07-27; Cosmetics Regulation: REGULATION (EC) No. 1223/2009), European  
163 substances legislation No. 1907/2006 (Registration, Evaluation, Authorization and Restriction of  
164 Chemicals (REACH)) in the European Union; and Section 4(h) (Reduction of Testing in Vertebrates) of the  
165 Toxic Substances Control Act (TSCA) in the United States. These regulations either prohibit the use of  
166 animal testing or only allow animal testing if results obtained by alternative methods are not sufficient  
167 to assess the sensitizing potential of a chemical. The “3Rs” together with the need for higher throughput  
168 and more mechanistically informative methods, continue to drive the development of non-animal  
169 methods. In this regard, *in silico*, *in chemico*, and *in vitro* methods in concert play an integral role in the  
170 hazard assessment of skin sensitization.

171 *In silico* models, along with *in vitro* tests, have been and continue to be developed for predicting the  
172 outcome of the four key events (KEs) described in the OECD adverse outcome pathway (AOP) for skin  
173 sensitization (OECD 2014). It is generally accepted that the skin sensitizing hazard of a chemical can be  
174 effectively assessed through the integration of non-animal approaches (Kleinstreuer et al., 2018; OECD,  
175 2017). However, there may be data gaps that are generated through the exclusion of chemicals that do  
176 not meet the physicochemical property requirements for the *in vitro* tests, and *in silico* methods that  
177 could be used to fill such gaps may lack transparency as they are sometimes viewed as “black box” tools.  
178 There is also no consensus on how to integrate *in vitro* data and/or *in silico* predictions for these events  
179 with existing *in vivo* data.

180 The protocol detailed in this publication outlines a framework in which *in silico* methods could be  
181 applied and integrated with existing *in vivo* and *in vitro* experimental data to identify potential skin

182 sensitizers, and to provide consensus on the development of models and the interpretation of model  
183 results. *In silico* methods are likely to play an important role in understanding the hazard and risk  
184 associated with chemicals (Myatt et al. 2018). Assessing sensitization is a necessary component of  
185 classification and labelling, workers' safety and occupational health (where ~20-30% of compounds may  
186 be sensitizers), regulation of cosmetics and other industrial chemicals as well as product discovery.  
187 Previous studies have evaluated the potential use of *in silico* tools to predict sensitization hazard or  
188 potential (Roberts and Aptula 2014; Roberts, Aptula, and Patlewicz 2006). However, there remains a  
189 need for *in silico* guidelines and the definition of principles and procedures that are specific to the  
190 prediction of skin sensitization relevant mechanisms. To this end, this skin sensitization protocol has  
191 been developed based on the experience of a cross-industry consortium comprising 39 different  
192 organizations and represents a consensus of how to use *in silico* methods to predict skin sensitization.

### 193 **1.1 Hazard Assessment Framework (HAF)**

194 Figure 1 provides a representation of a generic hazard assessment framework. The hazard assessment  
195 framework defines the relationship between mechanisms and effects that are relevant for the  
196 prediction of skin sensitization. The mechanisms and effects are molecular perturbations and  
197 manifestations, respectively, that lead to the adverse outcome and are reflected in the AOP for skin  
198 sensitization (Myatt et al. 2018). The mechanisms and effects are assessed based on *in silico* or existing  
199 experimental data. Each mechanism/effect assessment is assigned a reliability score which reflects the  
200 inherent quality of the assessment (Section 4). The relevance (scientific predictivity) of the  
201 effect/mechanism is also assessed. Rules and principles are used to combine the mechanisms/effects to  
202 derive an assessment of non-apical endpoints (i.e., endpoint 1 and 2 in figure 1) that are relevant for  
203 sensitization. The non-apical endpoint assessment is assigned a confidence score, which is a reflection of  
204 the reliability, relevance, and completeness of the assessment. Non-apical endpoints are combined via  
205 rules and principles to derive an overall assessment for skin sensitization (the apical endpoint) with an  
206 associated confidence score. The framework is designed to derive an assessment for hazard, with risk  
207 being outside the scope of the protocol. Figure 2 shows the hazard assessment framework for  
208 sensitization and the relationships between the following endpoints:

- 209 • Covalent interaction with skin proteins
- 210 • Events in keratinocytes
- 211 • Events in dendritic cells
- 212 • Skin sensitization *in vitro* (defined approach)

- 213 • Skin sensitization in rodent lymphocytes
- 214 • Skin sensitization in rodents
- 215 • Skin sensitization in humans (weight of evidence)

216 A comprehensive and mechanistic assessment for skin sensitization includes the four KEs described in  
217 the AOP as well as available *in vivo* data and other supporting elements (OECD 2014). A mechanistic  
218 understanding of the sensitizing process is detailed within the AOP for skin sensitization and becomes  
219 necessary in the development of this framework. In order for a chemical to exert a sensitizing effect, a  
220 series of well-defined stages/events occur that lead to the development of effector T cells (as opposed  
221 to regulatory T cells, which lead to tolerance(OECD 2014). A chemical's ability to induce each KE is  
222 critical information that is used in the development of the HAF. Sensitization is acquired through two  
223 distinct phases. During the initial induction phase, the immune system is primed through dendritic cell  
224 presentation of the sensitizing chemical to naïve T-cells. The induction phase occurs upon first contact  
225 with the sensitizer and a physiological response is typically mild or absent. Upon re-exposure to the  
226 same sensitizer, the primed immune system is activated and an inflammatory response occurs. This  
227 phase is called the elicitation or challenge phase and results in the manifestation of the symptoms  
228 associated with ACD: the appearance of rashes, blisters, and welts. A comprehensive assessment of the  
229 skin sensitization potential of a chemical includes the four KEs that are described in the induction phase  
230 (OECD 2014).

### 231 **1.1.1 Key Event (KE) 1: Molecular Initiating Event (MIE) – covalent interaction with skin** 232 **proteins**

233 The MIE for acquiring skin sensitization is the covalent binding of an electrophilic chemical to a  
234 nucleophilic protein, typically the thiol group of cysteine or the primary amine group of lysine (Figure 3).  
235 The interaction of the sensitizer (hapten) with the protein leads to the formation of a stable hapten-  
236 protein conjugate. While a hapten-bound protein may result from direct interaction of the protein with  
237 an electrophile, some chemicals require either metabolic (pro-haptens), or abiotic transformation  
238 through oxidation (pre-haptens) prior to complexing with dermal proteins. The hapten-protein  
239 interaction depends on the number of available nucleophilic target residues, steric considerations  
240 (targets on the surface of a protein are more easily accessible than those in folds), and the  
241 microenvironment (hydrophilic or hydrophobic)(OECD 2014). The formation of this complex is critical for  
242 the activation of the immunological cells that are responsible for sensitization.

### 243 **1.1.2 Key Event (KE) 2: Events in keratinocytes**

244 It is accepted that interactions with the hapten lead to the modulation of inflammation-related  
245 pathways and oxidative stress response pathways in keratinocytes (OECD 2014)(Figure 3).

246 Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that trans-locates into the  
247 nucleus of keratinocytes and binds to antioxidant/electrophile response elements (ARE). This in turn,  
248 initiates the transcription of genes related to oxidative stress responses, such as NADPH-quinone  
249 oxidoreductase 1 (NQO1) and glutathione S-transferase (GST). Nrf2 is repressed and controlled by the  
250 Kelch-like ECH-associated protein 1 (Keap1), which facilitates the ubiquitination and degradation of  
251 Nrf2. Keap1 is a cysteine (thiol) rich protein which can be modified by electrophiles (haptens) and  
252 oxidants. This modification to Keap1 induces conformational changes in the protein that releases bound  
253 Nrf2, allowing it to bind AREs and promote the expression of cyto-protective mechanisms (OECD, 2012).  
254 In addition, interaction of the hapten with keratinocytes stimulates the production of pro-inflammatory  
255 cytokines such as IL-18 (Natsch 2010). The release of cytokines by keratinocytes (among other factors)  
256 plays a role in stimulating the maturation of dendritic cells (Sumpter, Balmert, and Kaplan 2019)

### 257 **1.1.3 Key Event 3: Events in dendritic cells**

258 Langerhans cells and dermal DCs are responsible for the presentation of the protein-hapten complex to  
259 naïve T-cells in the lymph node during the induction phase (Figure 3). Following the uptake of the  
260 protein-hapten conjugate, DCs process and present these peptide fragments in the context of major  
261 histocompatibility complex (MHC) molecules to naïve T cells. Matured DCs migrate to the dermis and to  
262 the lymph node under the influence of cytokines and chemokines that are secreted by keratinocytes and  
263 fibroblast in the dermis (OECD 2014; Sumpter et al. 2019). During maturation, cell surface markers,  
264 adhesion molecules, cytokines, and chemokines are upregulated. The upregulation of co-stimulatory  
265 adhesion molecules (e.g., CD54, CD86) ensures that professional antigen presenting cells develop and  
266 initiate an immune response. When there is a lack of co-stimulation, T-cell anergy (a state in which the  
267 lymphocytes remain hypo-responsive after encounter with antigen) and a lack of sensitization may  
268 result (OECD 2014; Vocanson et al. 2009)

### 269 **1.1.4 Key Event 4: Events in lymphocytes**

270 Presentation of the fragmented peptide complex within the MHC to naïve T-cells results in their  
271 activation. This leads to the differentiation and proliferation of memory T-cells. Memory T-cells migrate  
272 to the dermis and also circulate throughout the body. Upon re-exposure to the same hapten, the

273 memory T-cells are activated (elicitation phase) and the immune response is triggered; the result is the  
274 manifestation of ACD, an irreversible immunologic response (OECD 2014).

275 KE 1-4 can be used to assess the 'skin sensitization *in vitro* endpoint', which in turn can be extrapolated  
276 to the 'skin sensitization in humans' endpoint as shown in Figure 2. These *in vitro* endpoints can also be  
277 predicted by *in silico* models as outlined in the HAF (Figure 2) and described in Section 2.

278 The availability of *in vivo* (usually rodent) data is relevant to the overall assessment of 'skin sensitization  
279 in humans' and facilitates the development of *in silico* methods to predict the results. KE 4 (lymphocyte  
280 activation and proliferation) can be measured with an *in vivo* mouse model and the adverse outcome  
281 (e.g., erythema) can be assessed in guinea pigs. The events in lymphocytes (when assessed in mice) and  
282 the guinea pig assessments can be combined to provide an overall assessment of 'Skin sensitization in  
283 rodents'. Skin irritation may be a confounding factor and so is also considered at this point. An overall  
284 assessment of 'skin sensitization in humans' can be determined through the integration of the 'skin  
285 sensitization *in vitro*' and 'skin sensitization in rodents' endpoints. Historical human test data may also  
286 be available and *in silico* models can be developed to facilitate its prediction. This information also  
287 propagates into the 'skin sensitization in humans' endpoint.

288 The HAF consists of evaluation of KE1-4 via *in vitro* or *in vivo* testing, physio-chemical properties, and  
289 human data (Figure 2). The assumption is made that all chemicals are capable of dermal penetration as  
290 a conservative measure (Fitzpatrick, Roberts, and Patlewicz 2017). The endpoints in the framework may  
291 be informed through available data, *in silico* predictions, or data acquired through conducting a test. The  
292 protocol defines general rules and principles for integrating data towards an overall prediction of the  
293 adverse outcome in humans. The incorporation of lines of evidence that may not directly relate to  
294 sensitization; such as skin irritation, means that the protocol takes the form of an integrated approach  
295 to testing and assessment (IATA).

## 296 **1.2 Integrated approach to testing and assessment (IATA)**

297 Given the definition of an AOP for skin sensitization and the availability of historical data, the endpoint is  
298 effectively predicted using an IATA. Limited data for the KEs along the AOP have restricted the  
299 development and applicability of *in silico* models to predict these endpoints while *in vitro* testing is  
300 mainly used to derive an assessment of the activation of KEs along the AOP pathways. This may change  
301 in the future, as more data become available and more robust *in silico* models can be developed.  
302 Nonetheless, through an integrated scheme, the overall endpoint of 'skin sensitization in humans' is

303 assessed as a function of the activity at each KE, with additional evidence from either existing data or *in*  
304 *silico* predictions of *in vivo* responses and metabolic biotransformation. Previous research has focused  
305 on developing such schemes and these non-animal integrated strategies are receiving interest from  
306 regulatory authorities. The publication of the 'Interim Science Policy: Use of Alternative Approaches for  
307 Skin Sensitization as a Replacement for Laboratory Animal Testing' is an example of regulators adopting  
308 this more integrated approach(EPA 2018). Additional non-animal assessment strategies are currently  
309 being developed and validated, and more approaches may be adopted for regulatory purposes in the  
310 future (Kleinstreuer et al. 2018). While several integrated approaches invoke the AOP and integrate the  
311 KEs to derive an overall assessment of skin sensitization, it has been argued that failure or ability to  
312 sensitize could be explained by (in)sufficient activity in the 'covalent interaction with skin proteins'  
313 endpoint, and the evaluation of subsequent KEs is less important (Roberts and Aptula 2008). To this end,  
314 the authors believe that a HAF that can facilitate multiple approaches is necessary. The ideal framework  
315 should be generic enough to facilitate possible variations in analysis while maintaining a high level of  
316 reproducibility and transparency. Rules and principles for combining results for each endpoint are  
317 defined in this protocol. These rules will set the foundation for the reproducibility and flexibility of the  
318 framework presented here.

### 319 **1.3 Defined Approaches**

320  
321 Previous approaches have incorporated rules that connect various aspects of the toxicological pathway  
322 to skin sensitization. The "2 out of 3" integrated testing strategy approach to skin sensitization hazard  
323 identification proposed by BASF uses a data interpretation procedure (DIP) that labels a chemical as a  
324 sensitizer or non-sensitizer based on the concordant reactivity of the chemical in two *in vitro* tests for  
325 KE1 - KE3 (Urbisch et al. 2015). Several other integrated strategies have been developed to assess either  
326 hazard or potency (Section 1 of the supplementary materials and described in detail elsewhere (OECD  
327 2017)). Each approach addresses particular elements of the AOP. At the time of this manuscript, no  
328 single approach is viewed as being superior to the others and selected approaches vary based on the  
329 availability of computational tools and data.

### 330 **1. *In silico* methodologies and models**

331 Historically, *in silico* models have focused on the prediction of animal data (particularly the LLNA), and  
332 few have considered the rest of the mechanisms established in the AOP. Therefore, it is necessary to  
333 examine how *in silico* tools could be developed to model mechanisms related to the KEs described  
334 earlier.

335 Depending on the availability of high-quantity data, different types of *in silico* models can be developed.  
336 Table 1 provides a list of data sources. Larger amounts of data, preferably with a strong mechanistic  
337 understanding of a specific toxicological process, can support many different types of models. Datasets  
338 that cover a broad chemical space can support the development of global Quantitative Structure-  
339 Activity Relationship ((Q)SAR) models, provided that the descriptors are relevant and mechanistically-  
340 related to the endpoint that is being predicted (Roberts et al. 2007). Where data are sparse, generated  
341 with different protocols, or generated through multiple mechanistic pathways (as may be the case in  
342 human studies), methods such as expert-alerts or read-across may be more appropriate.<sup>1</sup> Statistical  
343 models may also be developed; however, these models are potentially limited by a smaller applicability  
344 domain. On the other hand, the mechanistic understanding and classification of chemicals into a  
345 mechanistic domain means that local QSAR modeling may be a feasible approach for assessing events  
346 related to the sensitizing endpoint. One of the earliest attempts to develop a local mechanism-based  
347 QSAR model to predict EC3 concentrations in the LLNA, used the Relative Alkylation Index (RAI, a  
348 function of electrophilic reactivity, lipophilicity, and dose)(Roberts et al. 1991; Roberts and Williams  
349 1982). Subsequently, several Quantitative Mechanistic Models (QMM) have been developed with the  
350 goal of identifying physicochemical and other descriptors that contribute to a mechanistic  
351 understanding of an endpoint of interest (Aptula and Roberts 2006; Roberts and Aptula 2014; Roberts,  
352 Aptula, and Patlewicz 2011; Roberts and Natsch 2009). The rest of Section 2 discusses the mechanisms  
353 or effects that could be predicted and which types of *in silico* methodologies could facilitate the  
354 predictions. On a general note, *in silico* methods typically derive structure activity relationships (SAR) for  
355 organic salts by using the structure of the freebase. In cases where a metallic fragment will be removed  
356 in the generation of the freebase to derive the SAR form of the structure, the potential hazard posed by  
357 the metal should be considered. In the area of skin sensitization, removing nickel fragments may lead to  
358 an underestimation of hazard for structures that contain them. To more accurately facilitate predictions  
359 in these cases, the metal may be attached to the ligand, or the metal may be kept unattached in the  
360 training set. The model builder may also decide to remove the salt structure entirely from the training  
361 set; thereby, excluding the metal from the applicability domain of the model.  
362 The following sections describe general considerations for building *in silico* models based on the  
363 available chemistry, biology, and testing data. Section 1 of the supplementary material provides a  
364 detailed description of the experimental data that are relevant for assessing skin sensitization. Methods

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<sup>1</sup> The reader is referred to (Myatt et al., 2018) for a more general discussion on these methods

365 to assess the reliability of the data as well as *in silico* predictions have been previously described by  
366 (Myatt et al. 2018) and are summarized in Section 2 of the supplemental material.

## 367 **2.1 Covalent interaction with skin proteins, KE1**

368 *In silico* and or experimental assessments for whether a given compound will participate in covalent  
369 interactions with skin proteins are primarily generated based on understanding of metabolism, reaction  
370 domain assignment and protein reactivity.

### 371 **2.1.1 Dermal Metabolism**

372 The allergenic potential of a chemical may be increased or decreased through metabolic pathways or  
373 abiotic oxidation; these factors are important for predicting a chemical's potential to induce dermal  
374 sensitization. Metabolic detoxification takes place in two phases, which may or may not occur  
375 simultaneously. Phase II metabolism appears to be more abundant and active in the skin than in the  
376 liver, although Phase I enzymes – though not dominant – are inducible in the skin (Dumont et al. 2015).  
377 Given differences in expression profiles between the liver and skin, the potential use of liver metabolic  
378 data to predict metabolites in the skin will necessitate strategies for accounting for the differences in  
379 the expression of isoenzymes between liver and skin (Madden et al. 2017).<sup>2</sup> One strategy for predicting  
380 metabolic activation towards sensitization in dermal tissues is to derive alerts to indicate if a chemical  
381 may be a pro-hapten. This approach is currently limited by the size of the databases of pro-haptens and  
382 a general lack of skin specific data (although knowledge has been gained through experience over the  
383 years). Currently, it appears that the range of structural features that are activated towards sensitization  
384 via metabolic pathways is small. Given the absence of skin-specific metabolic data, it is challenging to  
385 definitively conclude on the topic. (Natsch and Haupt 2013) investigated the activation of pro-haptens  
386 by rat liver S9 fractions in the KeratinoSens<sup>TM</sup> assay, and identified phenolic and alkoxy groups attached  
387 to a benzene ring, some aromatic amines, and conjugated dienes in or in conjunction with six-  
388 membered ring as structural features that may require pro-activation to behave as haptens in the assay  
389 (Natsch & Haupt, 2013; Bergström, et al., 2006; Bergström, et al., 2006). The features identified do not  
390 represent a comprehensive and thoroughly defined list of features that undergo metabolic  
391 transformation leading to sensitization.

---

<sup>2</sup> The supplemental material provides a brief summary of the differences between skin and liver metabolic enzymes with relevance to humans

### 392 2.1.2 Reaction Domain

393 Existing mechanistic information on hapten-protein interactions has been used to construct *in silico*  
394 models for predicting sensitization potential based on a compound's structure and known – or predicted  
395 – reaction chemistry. The mechanisms for forming protein-hapten complexes involve the interaction  
396 between an electrophilic chemical (hapten) and the nucleophilic moiety on a skin protein (generally thiol  
397 or primary amine groups). Common mechanisms by which the sensitizer (hapten) may bind to the  
398 protein are: Michael addition, acylation, Schiff base formation, unimolecular nucleophilic substitution  
399 (SN1), bimolecular nucleophilic substitution (SN2), or nucleophilic aromatic substitution (SNAr). Within  
400 each of these mechanistic domains, there are mechanistic alerts and structural alerts. Structural alerts  
401 are defined as molecular substructures that can activate the toxicological effect or mechanism (Myatt et  
402 al. 2018). Structural alerts that are characterized by a common reaction site are defined as mechanistic  
403 alerts (Aptula and Roberts 2006; Enoch, Madden, and Cronin 2008; Roberts et al. 2015). Structural and  
404 mechanistic data do not always suggest a toxic effect, however – some structural features, such as steric  
405 hinderance, have been found to mitigate toxicity by decreasing the ability of the hapten to covalently  
406 bind to proteins – and these features may improve an *in silico* model by providing this additional  
407 information.

408 Classification of mechanistic and structural alerts within mechanistic domains allows for local QSAR  
409 modelling within each domain (OECD 2011), provided that one has the relevant quantitative information  
410 describing the protein-hapten bond. To this end, the following physical-chemical property descriptors  
411 are commonly used to predict interactions between haptens and proteins: Molecular weight (MW), Log  
412 P, solubility, rotational bonds, electronic and topological descriptors (e.g., quantum mechanics  
413 calculations), or chemical structure-based descriptors (e.g., the presence or absence of different  
414 functional groups) (OECD 2011). The factors constituting an acceptable and validated model have been  
415 described in previous work (Myatt et al. 2018). However, it must be noted that due to the expert nature  
416 of deriving structural alerts based on reaction chemistry, existing *in silico* tools can only incorporate our  
417 current knowledge of protein-hapten reaction chemistry (rather than the quantification of a physical or  
418 biological process), and that future models could be improved as we increase our mechanistic  
419 understanding of these processes. QSARs on the other hand, are not limited by current knowledge of  
420 mechanistic processes and the combined use of structural alerts and QSARs may add value to the  
421 analysis.

### 422 **2.1.3 Protein Reactivity**

423 Protein reactivity has been studied using model nucleophiles to assess protein-chemical interaction in *in*  
424 *chemico* assays. While the binding mechanism between the protein and the chemical could be described  
425 based on reaction chemistry as discussed in the previous section, any *in silico* tools (either statistical or  
426 expert rule-based) developed based on *in chemico* assay data will be limited in their ability to predict  
427 sensitization due to pro-activation. To overcome this limitation, predictions based on reaction  
428 chemistry, protein reactivity, and dermal metabolism should be considered in concert to generate an  
429 overall assessment (described in Section 3.1).

430 While protein reactivity measurement is feasible across all reaction domains described in section 2.1.2,  
431 experimental results show that within the domain of Schiff base formers there is a lower correlation  
432 between the *in chemico*-based DPRA model and *in vivo* and human data (Urbisch et al. 2015). While  
433 Schiff base formation may be theoretically feasible, the abundance of water within the peptide  
434 reactivity testing environment may limit some reactions. As such, peptide reactivity was found to  
435 correlate poorly with the potency of aldehydes, as Schiff base formation may be limited under testing  
436 conditions in the DPRA (Natsch et al. 2015). Further analysis revealed that more potent Schiff base  
437 formers (atranol, chloratranol, and salicylaldehyde) are reactive under physiological conditions (Natsch  
438 et al. 2012). However, the LLNA EC3 values of Schiff base formers are well correlated ( $R^2 = 0.95$ ) with a  
439 combination of logP and a reactivity parameter based on substituent constants (Roberts et al. 2006).  
440 Differential reactivity within a mechanistic domain is an issue that could become relevant in the  
441 development of *in silico* models, and particularly in those that use read-across. Such instances may not  
442 be unique to the protein reactivity mechanism but may require examination across all toxicological  
443 endpoints.

### 444 **2.2 Events in keratinocytes, KE2**

445 A comprehensive prediction of keratinocyte activation covers events on several levels of biological  
446 organization and includes the expression of biochemical, genomic, and proteomic pathways, and  
447 quantifies the release of pro-inflammatory mediators that stimulate dendritic cells in KE3 (OECD 2014).  
448 Validated protocols are established for assessing the induction of ARE dependent pathways, and, as  
449 such, the development of *in silico* models can be considered for this assessment. However, the breadth  
450 of information and data describing other pathways could be informative and may drive the development  
451 of *in silico* models to predict additional pathways in the future.

452 Statistical modelling is feasible; however, the availability of data is a critical factor influencing the  
453 success of measures to implement models based on AOP *in vitro* tests. Descriptors relating to the  
454 covalent modification of the cysteine-rich Keap1 protein could be used to develop mechanistically-  
455 relevant QSAR models. There may be limitations in predicting compounds which preferentially bind  
456 hard nucleophiles such as lysine since the *in vitro* tests predicting KE2 rely on the cysteine-dependent  
457 modification of Keap1. Therefore, false negative predictions may be more common for compounds that  
458 react via acyl transfer, within the domain of Schiff base formers, including short chain aldehydes, and  
459 longer chain saturated alkanals. Other electrophiles that prefer hard nucleophiles may also produce  
460 false negative predictions (Urbisch et al. 2015). This could be a potential issue in read-across analysis  
461 and should be addressed during an expert review.

462 *In silico* prediction of KeratinoSens™ and LuSens (*in vitro* test methods for assessing ARE activation in  
463 keratinocytes) data yields dichotomous (either positive or negative) test results (OECD 2018b). However,  
464 integrated assessments of potency may require continuous data input such as EC<sub>1.5</sub> (the lowest  
465 concentration inducing a 1.5-fold change in luciferase activity), IC<sub>50</sub> (concentration for 50% reduction of  
466 viability) and EC<sub>3</sub> values (concentration with 3 fold luciferase induction) (Natsch et al. 2015).

### 467 **2.3 Events in dendritic cells, KE3**

468 Dendritic cell activation is similar to keratinocyte activation in that predictions can be made on the levels  
469 of protein and gene expression. Methods have been validated for measuring the expression of specific  
470 cell surface markers which contribute to T cell activation and proliferation. Published databases may  
471 contain data for dendritic cell gene expression of co-stimulatory and adhesion molecules (cell surface  
472 markers: CD54 and CD86) and Interleukin-8 (IL-8) (Nukada et al. 2011; Urbisch et al. 2015).

473 As noted for the KE2 endpoint, care must be taken when integrating testing data from the various *in*  
474 *vitro* assays into KE3 *in silico* models due to differences in the types of data that may be produced by  
475 different assays. The continuous data outcomes predicted for these assays, such as the EC<sub>150</sub> and EC<sub>200</sub>  
476 values from the h-CLAT assay; the CV<sub>70</sub> and the EC<sub>150</sub> in the U-SENS™ assay could be used in integrated  
477 strategies to predict potency. These and other *in silico* predictions of the Ind-IL8LA (induced interleukin-  
478 8 luciferase activity) could be used to support the hazard assessment; however, since a statistically-  
479 derived experimental variable (confidence interval) is needed to determine a positive call, a more  
480 practical approach may be to dichotomize the assay results and make binary predictions.

481 Often, it is helpful to build models that use threshold values to convert continuous data into  
482 dichotomous (yes or no) values. For any of the *in vitro* or *in chemico* test methods that are used to  
483 assess a KE along the AOP, using threshold values, *in silico* predictions could generate dichotomous  
484 predictions of KE activity using these *in vitro* or *in chemico* test endpoints.

#### 485 **2.4 Events in human lymphocytes, KE4**

486 The lack of standardized data makes *in silico* predictions of *in vitro* T cell activation and proliferation  
487 challenging. A paucity of data for this endpoint is not surprising, however, as the value of predicting this  
488 key event remains in question, and the significance of an *in vitro* estimate of KE4 can only be speculated  
489 at this time. It is possible that the magnitude of the T cell responses at KE4 may be the key event that  
490 allows us to make distinctions between different potency classes *in vitro* (OECD 2014), but the issue has  
491 not been settled. Consequently, only the *in vivo* Local Lymph Node Assay has been accepted as a  
492 standardized method for assessing this endpoint.

#### 493 **2.5 Events in rodent lymphocytes, KE4**

494 The LLNA is the only standardized *in vivo* method used to measure the proliferation of lymphocytes in  
495 response to immune system priming by a test chemical as well as the potency of the chemical as a skin  
496 sensitizer. The results of the assay are reported as the concentration of the chemical needed to induce  
497 T-cell proliferation by a pre-chosen factor (usually 3, 1.6, or 1.8 times the baseline amount as assessed  
498 by the stimulation index (SI))(OECD 2010b, 2010a, 2018a). The LLNA has been used extensively, and it is  
499 quite feasible to build *in silico* models using statistical and rule-based methods due to the ready  
500 availability of data, although, the majority of such data is proprietary. While the publicly-available LLNA  
501 data could facilitate statistical modeling, the model coverage may be reduced for industrial applications.  
502 However, the combined use of statistical modeling and structural alert definitions could be a strategy to  
503 overcome this limitation.

504 The irritation potential of a chemical could be a confounding factor in the experimental LLNA, and the  
505 issue of irritation translates into *in silico* assessments. Training set examples and analogs under  
506 consideration for read-across should be examined for their irritation potential. Studies indicate that non-  
507 sensitizing irritants (such as surfactants) could be overestimated by the LLNA, leading to false positive  
508 results (Ball et al. 2011; OECD 2010a). While this is certainly the case for sodium lauryl sulfate (SLS),  
509 chloroform/methanol, Triton X-100, oxalic acid, methyl salicylate, and nonanoic acid, analysis of  
510 chemicals known to be skin irritants has not validated this generalization across the entire class of non-  
511 sensitizing irritants(Ball et al. 2011). Most non-sensitizing irritants are negative in the LLNA and those

512 that are positive may produce borderline results (with few exceptions). For example, the sensitization  
513 hazard of SLS is derived from a clear dose-response curve that is indicative of a positive LLNA result;  
514 however, when a weight-of-evidence (WoE) approach is used, the interpretation of the LLNA results may  
515 be reversed. There is no evidence that SLS is a skin sensitizer in humans despite exposure; albeit limited,  
516 it lacks a structural alert for sensitization and is a strong irritant (Basketter et al. 2009). Hence, Basketter  
517 et al. 2009 have suggested that for the SI results obtained for SLS in the LLNA ( $SI_{SLS}$ ), a WoE approach  
518 could be developed around the false positive result to implement this approach in a general sense.  
519 Using SLS as reference for a test chemical with unknown skin sensitization hazard, irritant potential and  
520 SI predictions ( $SI_{test}$ ); if the  $SI_{test} < SI_{SLS}$  and no structural alert exists of sensitization, then the LLNA  
521 prediction could be a suspected false positive and confidence in a positive prediction of the “skin  
522 sensitization in humans” endpoint is low. The reverse may also be considered: If  $SI_{test} > SI_{SLS}$  and an  
523 alerting structure exists for sensitization; then the chemical may be suspected to be a true positive  
524 (Basketter et al. 2009). The confidence could be adjusted accordingly based on the weight of evidence  
525 presented. This sort of analysis would be considered with a low reliability LLNA study which may have  
526 been conducted at irritant concentrations. Generally, the LLNA test is preceded by dose finding range  
527 studies and minimally irritating to not irritating concentrations are tested.

528 Some LLNA protocols (LLNA-DA, and LLNA-BrdU-ELISA) use non-radioactive methods to quantify  
529 lymphocyte proliferation. Results from these protocols could be combined in training sets that would  
530 facilitate binary level predictions; however, varying criteria for predicting a positive call may complicate  
531 the prediction of a meaningful continuous SI or  $EC_x$  value (where x is 3, 1.6, or 1.8 depending on the  
532 LLNA protocol used) from such a dataset and would require a valid strategy for integrating the data.  
533 Another relevant issue with LLNA datasets that arises in the curation process is the comparison and  
534 combination of SI and  $EC_3$  values for tests conducted in different vehicles. While it seems logical that  
535 vehicle effects are normalized in the derivation of the SI and  $EC_3$  values, there are mechanisms that  
536 could lead to enhanced bioavailability depending on the choice of vehicle. The rapid evaporation of  
537 acetone, for example, may result in volatilization of the test chemical and decreased bioavailability;  
538 whereas dimethyl sulfoxide (DMSO) could potentially enhance penetration. Differing results may be  
539 obtained between two LLNA tests using different vehicles and this could influence hazard assessment  
540 (Hoffmann 2015). In some cases, vehicle effects may lead to the assignment of a chemical to two  
541 neighboring potency classes (Anderson, Siegel, and Meade 2011; Basketter, Gerberick, and Kimber 2001;  
542 Dumont et al. 2016; Hoffmann 2015). This inherent variability in the LLNA data (not exclusively caused  
543 by different vehicles) is translated to *in silico* predictions. When combining multiple data sources, the

544 most conservative SI and EC<sub>x</sub> values could be adopted, unless there is compelling evidence that the  
545 vehicle is potentiating or attenuating the effect of the test chemical. A less conservative, but valid,  
546 approach is to use the mean, or median values, among other valid approaches (Hoffmann et al. 2018).

## 547 **2.6 Skin sensitization in rodents**

548 The skin sensitization in rodent endpoint is evaluated through the use of the GPMT and the BT method.  
549 Guinea pigs were historically used to assess skin sensitization. Similar to the LLNA, while public data are  
550 available, much of the GPMT and BT data are proprietary. The data that exists could facilitate statistical  
551 modeling, the derivation of expert alerts, and read-across.

## 552 **2.7 Skin sensitization in humans**

553 Historical data exist for this endpoint and, based on data quantity, expert-alert derivation and read-  
554 across may be preferable to statistical methods. *In silico* predictions could be useful for the prediction of  
555 dichotomized results of positive/negative. Potency predictions could be challenging based on data  
556 availability. Evidence to support human predictions includes clinical data (DPT) and usage/occupational  
557 exposure data (Api et al. 2017). Further, the integration of the 'skin sensitization *in vitro*' and the 'skin  
558 sensitization in rodents' endpoints, along with any direct human evidence, are considered together as  
559 weight of evidence for the prediction of the 'skin sensitization in humans' endpoint.

## 560 **3. Endpoint assessment and confidence**

561 The protocol details the integration of data with different reliabilities and relevance. Further, there may  
562 be cases in which information that is critical to an assessment is missing. This section outlines the  
563 rules/principles that could be applied when deriving an assessment and its associated confidence based  
564 on the totality of evidence presented. Figure 4 shows the hazard assessment framework annotated with  
565 references to where each of the following sections applies.

### 566 **3.1 Covalent interaction with skin proteins assessment**

567 Assessment of the 'covalent interaction with skin proteins' endpoint includes consideration of metabolic  
568 transformation, reaction chemistry, and DPRA/ADRA predictions. Figure 5 shows how rules could be  
569 made around the available information to derive an overall prediction of hazard. If an experimental  
570 result is positive for the methods assessing KE1 (DPRA/ADRA), then a positive assessment of the  
571 'covalent interaction with skin proteins' is warranted. However, the reliability of the prediction, as  
572 assessed by the scheme presented in Table 6 of the supplementary material and described in (Myatt et  
573 al. 2018), varies depending on the quality of the information presented and this has an influence on the

574 confidence score. The quality and reliability of an *in silico* DPRA/ADRA prediction could be assessed  
575 according to the expert review criteria described in (Myatt et al. 2018). Additional considerations for  
576 both experimental (test article) and *in silico* (training set examples and analogues) results include  
577 situations in which DPRA/ADRA could lead to a false positive result due to oxidizing properties of the  
578 test chemical, which can lead to peptide dimerization. An expert review could inform on whether or not  
579 this is likely and if the assessment and confidence score need adjustment. Assessments of negative  
580 DPRA/ADRA results vary based on consideration of the metabolic potential of the chemical together  
581 with knowledge of reaction chemistry. In general, when the chemical is expected to be out of the  
582 metabolic domain of the DPRA/ADRA then precedence is given to clearly-defined knowledge of reaction  
583 chemistry (including mitigating factors, such as sterics) in the overall assessment of the 'covalent  
584 interaction with skin proteins' endpoint. If the reaction chemistry indicates a mechanism leading to  
585 sensitization; particularly if the mechanism requires pro-activation then the overall assessment of the  
586 'covalent interaction with skin proteins' is positive based on reaction chemistry knowledge, but the  
587 confidence is medium. If the test article is out of the metabolic domain, negative in DPRA/ADRA and no  
588 mechanistic alert could be identified in the structure of the test chemical based on reaction chemistry,  
589 then the DPRA/ADRA result is inconclusive as it cannot be said that the overall assessment is either  
590 negative or positive. However, if metabolism is not predicted to occur and the chemical is considered  
591 within the metabolic domain of the DPRA/ADRA, then the negative result should be given consideration  
592 in the overall assessment. A negative DPRA/ADRA prediction (within the DPRA/ADRA metabolic domain)  
593 and a positive mechanistic alert lead to a negative overall assessment, with a medium confidence level,  
594 given that the DPRA/ADRA result is experimental and the positive mechanistic alert introduces some  
595 uncertainty. An expert review would consider whether or not the test chemical is within the Schiff base  
596 reaction domain. In these cases a negative DPRA/ADRA result may be mechanistically justifiable due to  
597 the protein-hapten interaction being unfavorable under the test conditions as a result of the abundance  
598 of water; particularly for chemicals that are indicated as less potent sensitizers by other methods. In this  
599 case, the overall assessment could be considered positive (after expert review) with a low confidence.  
600 This positive result is based on giving greater precedence to the mechanistic alert within this domain,  
601 and the decreased relevance of the DPRA/ADRA due to the differential reactivity of chemicals within the  
602 Schiff base domain. Further, co-elution of the test article with the model nucleophile may lead to false  
603 negative predictions, although this occurs to a lesser extent in the ADRA than in the DPRA (Fujita et al.  
604 2019).

605 In cases where the DPRA/ADRA result is positive, but no mechanistic alert can be assigned, it is worth  
606 considering whether mechanistic knowledge could be provided by the protein reactivity results  
607 particularly when close analogs point to the same structure-activity relationship. Figure 5 shows the  
608 'covalent interaction with skin proteins' endpoint and the confidence score decision tree based on RS1  
609 data. The confidence scores are expected to vary based on reliability and relevance; as such, there are  
610 several possible permutations of the decision tree. These general "rules" are expanded to provide a  
611 sense of the confidence assigned to assessments with varying reliabilities and relevance, Supplementary  
612 Material, section 4 (SM 4).

613

### 614 **3.2 Events in keratinocytes**

615 The confidence score obtained for the activation of the events in keratinocytes towards skin  
616 sensitization varies based on the Log  $K_{ow}$  of the chemical. If there is a positive prediction (RS1,  
617 experimental) and the Log  $K_{ow}$  is  $<5$ , then the result is assigned a high confidence. If the Log  $K_{ow}$  is  
618 greater than 5, then the confidence is medium for a positive result and low for a negative prediction,  
619 since limited information is available for such chemicals (OECD 2018b). Regardless of Log  $K_{ow}$  values,  
620 negative results could be further assessed based on the occurrence of metabolism and the chemical  
621 mechanism of action.

622 A metabolic alert (indicative of an expected metabolic transformation) along with a negative RS1/2  
623 experimental or RS3 *in silico* result, could indicate reduced relevance of the *in vitro* assays predicting KE2  
624 in this case – possibly because limited metabolic competency of the cells used in the assay are  
625 responsible for a false negative. Therefore, the overall assessment would be negative but with a low  
626 confidence score. If there is no biochemical transformation predicted, then the chemical mechanism of  
627 action could be considered. A negative assessment for a chemical within the acyl transfer domain and  
628 Schiff Base domain is conservatively assigned a low confidence score based on the preference of  
629 chemicals within these domains for the lysine instead of the cysteine moiety (representing decreased  
630 relevance). It is worth mentioning that some chemicals within these domains are accurately predicted as  
631 true negatives and a review of the relevance is necessary to assign a higher confidence. Such a review  
632 might include an examination of close analogs (or the test structure if data is available) for their  
633 assessment in the DPRA/ADRA and or an animal model. If close analogs are positive in the DPRA/ADRA  
634 and the lysine moiety; but not cysteine, is implicated for covalent modification then the relevance of the  
635 KE2 assays for predicting the test structure may be challenged. However, if cysteine modification is  
636 apparent in the DPRA/ADRA (positive for covalent interaction with skin proteins), it is more difficult to

637 challenge the relevance of the KE2 assays on that basis and conflicting information is presented by the  
638 two KEs. The analogs may be further assessed and screened for existing animal data and/or *in silico*  
639 predictions of the LLNA or GPMT. This serves the purpose to assess the likelihood of a false negative  
640 prediction of the test structure by the KE2 assays. Where a false negative seems likely, the low  
641 confidence is appropriate. In cases where the analogs are true negatives, the confidence score could be  
642 increased to a medium level and this reflects that while uncertainty is somewhat reduced, there is not  
643 absolute certainty in the assessment. Within any other domain, a negative KE2 prediction is considered  
644 with high confidence, given RS1/2 data. Varying reliabilities of the data could change the confidence  
645 scores in figures 6A and B (see SM Table 9).

### 646 **3.3 Events in dendritic cells**

647 An overall assessment of the events in dendritic cells could be made based on the h-CLAT (Figure 7), U-  
648 SENS™ or IL-8 Luc assays (Figure 8). A positive response from these assays typically translates to a  
649 positive overall call for the events in dendritic cells with high confidence in the activation of the  
650 dendritic cells towards sensitization, but an expert reviewer would be needed to adjust overall calls and  
651 confidence scores for certain chemical classes, structural features, and physical-chemical properties. For  
652 example: some chemical classes, such as surfactants, may lead to false positive results in the U-SENS™,  
653 and a negative result for a chemical that has a Log  $K_{ow}$  greater than 3.5 is considered inconclusive for the  
654 h-CLAT. The pro/pre-hapten status of the test chemical is also relevant in each of the three assays.  
655 Negative results for structures in which a site of metabolism leading to sensitization has been identified  
656 are accepted with a medium level confidence from the h-CLAT, U-SENS™ and IL-8 assays. In cases where  
657 there are no additional parameters confounding the prediction, then the confidence level is high for the  
658 negative predictions from the h-CLAT, U-SENS™, and IL-8 Luc assays.

### 659 **3.4 Skin sensitization *in vitro***

660 Integrating data to derive an overall assessment for the 'skin sensitization *in vitro*' endpoint that  
661 correlates with the *in vivo* endpoint is an active area of research. A number of defined approaches (DA)  
662 which use varying DIPs have been developed to determine an overall assessment of skin sensitization  
663 using non-animal/*in-vitro/in silico* models. Any of the DAs described in Section 1 may be adopted here.  
664 There has been regulatory acceptance of the "AOP 2 out of 3" approach and the KE3/1 sequential  
665 testing strategy (STS) as alternatives to the LLNA for regulatory submission to the United States  
666 Environmental Protection Agency (US EPA) (EPA 2018). Here, we discuss how to derive an overall

667 assessment and confidence when the “AOP 2 out of 3” approach is used within the framework  
668 presented in this protocol.

669 The “AOP 2 out of 3” uses the outcome of three individual assays that map to three KEs to derive a final  
670 assessment; however, within the framework presented the assay results are integrated and propagated  
671 to the three endpoints related to each key event. The difference between the “AOP 2 out of 3” and the  
672 approach used in the framework is subtle, but is worth mention. The “AOP 2 out of 3” approach  
673 considers the outcome of the experimental systems – DPRA, KeratinoSens<sup>TM</sup>, and h-CLAT – but within  
674 the framework presented, the: ‘Covalent interaction with skin proteins’, ‘Events in keratinocytes’, and  
675 ‘Events in dendritic cells’ (KEs in the AOP) are considered. These KEs are assessed based on knowledge  
676 of reaction chemistry and mechanistic understanding that is not explicitly considered within the “AOP 2  
677 out of 3” approach. Similar to the “AOP 2 out of 3,” an overall assessment of hazard for the ‘skin  
678 sensitization *in vitro*’ endpoint is determined based on a 2 out of 3 consensus among the endpoints. If  
679 outcomes (*in silico*/experimental) are available for only two endpoints, and they have aligned outcomes,  
680 the overall assessment of the endpoint is based on the concordant assessments and the lower  
681 confidence score propagates. The adoption of the lower confidence score reflects a conservative view of  
682 the assessment at this stage of the analysis. However, if the confidence scores have the same value for  
683 non-concordant assessments, then the overall prediction for the ‘skin sensitization *in vitro*’ endpoint is  
684 inconclusive. Where there are two concordant assessments, and the non-concordant assessment occurs  
685 with high confidence, then the overall confidence could be lowered by one level. Table 2 provides  
686 examples showing the derivation of the overall assessment and the rationale for the final confidence  
687 score. An alternative point of view suggests that the assays that predict the ‘events in keratinocytes’,  
688 and ‘events in dendritic cells’, are dependent on the ability of the test chemical to bind protein and  
689 therefore point to the activation of the molecular initiating event, ‘covalent interaction with skin  
690 proteins’. In this point of view, any improvement in predictive performance that results from  
691 integrating the KEs across the AOP is a result of reducing the influence of technical limitations of each of  
692 the assays (Roberts 2018; Roberts and Patlewicz 2018).

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703 The discussion thus far has focused on assessing hazard from *in vitro* data, but there are also existing  
704 strategies for predicting potency in humans from *in vitro* data based on the DAs described in Section 1  
705 and reviewed in (Kleinstreuer et al. 2018). The Artificial Neural Network Model for Predicting LLNA EC3  
706 (Shiseido); Bayesian Network DIP (BN-ITS-3) for Hazard and Potency Identification of Skin Sensitizers  
707 (P&G); Sequential Testing Strategy (STS) for Sensitizing Potency Classification Based on *in Chemico* and  
708 *In Vitro* Data (Kao); and ITS for Sensitizing Potency Classification Based on *In Silico*; *In Chemico*, and *In*  
709 *Vitro* Data (Kao) were found to predict potency class equally well, or better than the LLNA. Similar to the  
710 earlier discussion on hazard, the DAs for assessing potency use biological assay outcomes  
711 (mechanisms/effects assessment within the HAF e.g. DPRA, KeratinoSens™, h-CLAT) as endpoints and  
712 may integrate the information with *in silico* methods to determine a potency class. Within the HAF  
713 presented, the assay outcomes (*in vitro/in silico* effects/mechanisms assessment) are interpreted in the  
714 context of their toxicological significance and integrated to determine a toxicological endpoint according  
715 to the rules and principles outlined in previous sections. The overall assessments of the KE endpoints  
716 may substitute for the outcome of the individual test methods in data interpretation procedures.

### 717 **3.5 Skin sensitization *in vitro* to skin sensitization in human extrapolation**

718 Extrapolation of *in vitro* skin sensitization results to human skin sensitization predictions is necessary to  
719 satisfy the European Union's 7<sup>th</sup> Amendment of the Cosmetic Directive and REACH regulations which  
720 require and prefer the use of non-animal test methods for assessing the human skin sensitization  
721 endpoint. The definition of the AOP and the mechanistic information provided by the assays that map to  
722 the AOP allow the human hazard identified for the 'skin sensitization *in vitro*' outcome to be propagated  
723 to the human endpoint. The relevance of the integrated *in vitro* battery of tests is equally weighted with  
724 the *in vivo* studies except in unique cases; for example, when metabolism is thought to influence the  
725 outcome. As such, no change in confidence (reliability and relevance of the prediction) is expected due  
726 to the extrapolation of *in vitro* hazard.

### 727 **3.6 Skin sensitization in rodent lymphocytes**

728 A negative result in the LLNA is propagated to the skin sensitization in rodent lymphocytes endpoint  
729 with high confidence. A weak sensitizer may require investigation of the skin irritation potential of the  
730 chemical, particularly if the result is derived from a lower-reliability study that may not have considered  
731 irritation prior to designing the test. The skin irritation potential will be determined through a HAF that  
732 will be published in a separate protocol. Positive results due to confounding factors from irritants usually

733 result in a low-level increase in lymphocytes which could be misinterpreted as a weak sensitizing  
734 response. In cases where a chemical is found to have a strong skin irritation potential and is a weak  
735 sensitizer and the influence of irritation cannot be ruled out, a positive assessment with low confidence  
736 could be assigned to the 'Events in rodent lymphocytes' endpoint (Figure 9).

### 737 **3.7 Skin sensitization in rodents**

738 This endpoint integrates guinea pig (GPMT and BT) and mouse (LLNA) data. In the absence of LLNA data,  
739 the endpoint could be determined through the scheme shown in Figure 10. If guinea pig tests are not  
740 conducted according to standard protocols, irritation could become a confounding factor in the  
741 interpretation of the guinea pig test results and influence the relevance of the study (OECD 1992).  
742 Freund's complete adjuvant (FCA) is used to maximize the guinea pig response; however, FCA may also  
743 lower the irritation threshold. The implication is that concentrations that were identified as non-  
744 irritating and suitable for the challenge reaction might in fact produce an irritant response. Further, a  
745 hyperirritable state may be induced by the test article during the induction phase that is not  
746 represented in the control, unless a suitably irritating surrogate is used to induce the hyperirritable state  
747 in the controls (Kligman and Basketter 1995; OECD 1992). An irritant effect cannot be distinguished from  
748 an allergic response by visual examination. As such, post challenge examination is helpful in  
749 distinguishing a sensitization response from an irritant effect. Chemicals that are identified as irritants  
750 could be confidently predicted as non-sensitizers if observations of erythema dissipate within one day of  
751 challenge and/or there is a negative re-challenge test one week after the initial challenge (Kligman and  
752 Basketter 1995). A positive result for a chemical that is irritating but predicted to be a weak sensitizer is  
753 afforded a low confidence score if deviations from OECD 1996 result in decreased reliability and  
754 relevance of the study as discussed above.

755  
756 When both guinea pig and mouse data are available and are concordant, then the result is translated to  
757 the 'skin sensitization in rodent' endpoint with exact or higher confidence scores being adopted. For  
758 example, if the LLNA is positive with medium confidence and the GPMT/BT is positive with low  
759 confidence, then the skin sensitization in rodent endpoint is assessed as positive with medium  
760 confidence. In cases where the data are discordant, the strategy for deriving an overall assessment may  
761 vary case-to-case. A high reliability guinea pig test has an advantage over the LLNA because it includes  
762 both induction and challenge phases, and is as such, more representative of the entire sensitization  
763 process. However, in contrast to the LLNA, the guinea pig test results are based on a qualitative measure  
764 and a subjective endpoint. Potency is better assessed through the LLNA since it is derived from dose-

765 response relationships and the read-out is quantitative; nonetheless, some chemical classes are over-  
766 classified in the LLNA. It is valuable to consider how the challenge reaction affects interpretation of an  
767 assessment. It could be argued that the LLNA is an assay and non-specific reactions can occur that may  
768 or may not relate to allergenic potential (respiratory sensitizers test positive in the LLNA, for example)  
769 while the dermal challenge in the guinea pig tests lends more confidence that any observations of  
770 sensitization are specific to the skin. A default principle that could be adopted is to evaluate the 'skin  
771 sensitization in rodent' endpoint based on either the LLNA or GPMT/BT assessment with the higher  
772 confidence score and conservatively decrease the score by one level to reflect any uncertainty. For  
773 example, an LLNA that is assessed as positive with medium confidence, and a GP test that is negative  
774 with low confidence, would lead to a 'skin sensitization in rodent' assessment as positive with low  
775 confidence. In these circumstances, a review of the predictions is prudent and the assessment and  
776 confidence scores may be adjusted based on the review.

### 777 **3.8 Skin sensitization in rodents to skin sensitization in human extrapolation**

778 There are two schools of thought on rodent-to-human extrapolation that draw from a two different  
779 perspectives on risk assessment: one is that LLNA potency categories and EC3 values correlate well with  
780 human potency categories and NOEL values, and could therefore be used as a surrogate for the NOEL  
781 and for direct prediction of human potency class (Basketter et al. 2005). Alternatively, a safety factor  
782 may be incorporated based on the interspecies variation that may occur between the mouse and  
783 humans; although, this factor could be lowered in cases where a better correlation may be expected  
784 (e.g., based on existing human data for a close analogue) (Roberts and Api 2018). Roberts and Api 2018,  
785 have defined alerts for cases where the LLNA is not a good predictor of human potency. Guinea pig tests  
786 also provide relevant information on hazard and potency. However, tests that use adjuvant and  
787 intradermal routes of exposure (GPMT) present a challenge for interpreting human potency, and in  
788 those situations potency estimation via the BT may be more relevant. The data however, could serve in  
789 a weight-of-evidence case for potency determination through interpretation and comparison of  
790 different test results and also with known benchmark chemicals (Kimber et al. 2001).

### 791 **3.9 Skin sensitization in humans**

792 The 'skin sensitization in humans' endpoint could be evaluated through several other endpoints such as  
793 the 'skin sensitization *in vitro*' endpoint (section 3.5), the 'skin sensitization in rodent endpoints' (section  
794 3.8), or through the integration of the 'skin sensitization *in vitro*', 'skin sensitization in rodents', and  
795 human assessments, combined with supporting data from non-standard endpoints such as photoallergy.

796 A positive HMT/HRIPT is indicative of adverse outcome in humans and can potentially be used to assign  
797 a potency class. In the absence of reliable studies, other sources of evidence may be sought. The first  
798 line of evidence arises from the toxicological relationships that could be drawn from the chemical's  
799 structure. The presence of a structural alert for sensitization in humans provides evidence for the  
800 elicitation of the adverse outcome. Structural alerts and diagnostic patch testing with positive incidences  
801 in greater than 1% of the population (considered to be high incidence) in relation to low usage volume  
802 (a measure of exposure) provides evidence for the skin sensitization potential of a chemical, although it  
803 does not provide a definite assessment (Api et al. 2017). If a compound has no structural alerts and  
804 diagnostic patch testing data indicate < 1% frequency, the overall evidence may together indicate a  
805 negative assessment, especially if the use volume is high. It is important to note that the indication of a  
806 1% incidence rate is based on expert opinion and as such is not meant to represent a rule that requires  
807 strict compliance. Many combinations of scenarios are possible.

808 In cases where human and *in vitro/in vivo* sensitization assessments do not align, additional information  
809 could be gathered from the 'skin sensitization *in vitro*' and/or 'skin sensitization in rodents' endpoints to  
810 build a weight of evidence case. There are many permutations of assay results at this level but some  
811 general guidance can be provided to the evaluator towards an overall assessment. It is generally  
812 recommended that the assessments that are assigned more frequently should be propagated to the  
813 overall human endpoint. However, if reliable human data (RS1/2) is available, then the assessment of  
814 this data is given priority in the decision-making process. Table 13 of the supplementary material  
815 expands on the principles to derive an overall assessment given *in vitro* and rodent evidence. Due to  
816 ethical concerns, human testing is no longer considered appropriate for most compounds, so much of  
817 the human data is older, or based on clinical reports, and may therefore lack information to assess its  
818 quality, necessitating the filter of expert opinion. Careful consideration is required in assessing  
819 confidence of the HMT and HRIPTs. For the HMT and, especially for the HRIPT as used by the fragrance  
820 industry, low doses are often tested as the goal is to corroborate an animal study while trying to avoid  
821 sensitizing the subjects. Therefore, there can be quite a bit of uncertainty in a negative result because a  
822 higher test concentration could potentially produce a positive result in humans.

823 Table 3 shows factors to consider in assigning confidence to a human study in general. There are  
824 however some specific exceptions to these criteria when assessing the HMT and HRIPTs. The exposure  
825 scenarios in the HMT and HRIPT may not represent real-world exposure because the test chemical is  
826 applied under occlusive conditions and the outcomes can be viewed as subjective because an observer  
827 grades the skin reaction.

828

#### 829 4. Case Studies

830 The case studies demonstrate the interpretation of results when a series of statistical models ((Q)SARs),  
831 structural alerts, or read-across are used to fill data gaps for effects and mechanisms that are included in  
832 the hazard assessment framework. The studies demonstrate how aspects of the rules and principles are  
833 implemented to derive an assessment, reliability score and confidence score, when the assessment is  
834 made using either or both existing experimental data or *in silico* methods.

##### 835 4.1. Case 1a: Compound with conflicting data ("Skin Sensitization *in vitro*" endpoint 836 determination)

837 An assessor needed to determine the hazard associated with a compound. The compound was  
838 predicted to be reactive towards proteins via an Acyl or SN2 reaction, and could be assigned to a  
839 reaction domain based on reaction chemistry alerts. Data that was generated based on OECD TG 442C  
840 (DPRA) was available for the compound. The data indicated that the compound was negative for protein  
841 reactivity. Based on adherence to the test guideline, a reliability score of RS1 was assigned to the study.  
842 *In silico* tools (statistical results (QSAR) and alerts) were available for the DPRA prediction, and these  
843 predictions were also negative. The statistical model and the alerts both had a reliability score of RS5. *In*  
844 *silico* assessments of dermal metabolism were negative after an expert review. The review increased the  
845 reliability of the dermal metabolism alert from RS5 to RS3. The overall assessment for 'covalent  
846 interaction with skin proteins' was negative; however, the confidence was assigned as medium, based  
847 on the conflicting mechanistic/reaction chemistry alert for protein reactivity, Figure 11a.

848

849 There is experimental data for the KeratinoSens™ assay which is afforded a positive assessment with a  
850 reliability score of RS1 (the study adhered to OECD TG 442D), so the overall assessment for the 'Events  
851 in Keratinocytes' KE is positive with high confidence. Experimental data is not available for the 'Events in  
852 Dendritic Cells' KE. The assessor would like to use the "2 out of 3" approach and is faced with two  
853 conflicting assessments based on *in vitro* data. A statistical model (QSAR) was used to predict the results  
854 of the h-CLAT assay and the assessment is negative with a reliability score of RS3, after an expert review.  
855 The overall assessment of the 'Events in Dendritic Cells' KE is negative with a medium confidence. Based  
856 on the two concordant assessments with aligned confidence scores (Negative, Medium confidence), and  
857 a third assessment that is conflicting with high confidence (Positive, High confidence), the overall  
858 assessment of *in vitro* skin sensitization endpoint is negative with low confidence.

859 **4.2 Case 1b: Compound with conflicting data ('Skin Sensitization in Humans' endpoint**  
860 **determination)**

861 A further assessment was completed for the same compound as in Case 1a. This assessor has LLNA and  
862 GPMT data with conflicting assessments. The LLNA data is positive with an EC3 (%) value that indicates  
863 weak sensitization. The study is assigned the lowest reliability score of 5 based on significant deviations  
864 from OECD Test No. 429 that could alter both the reliability and relevance of the study. *In silico*  
865 assessments using expert alerts and statistical models are both negative. The weak sensitizing effect and  
866 the mis-aligned *in silico* results prompt the assessor to consider the irritation potential of the chemical.  
867 Experimental data is available for the *in vitro* skin irritation test using the Reconstructed Human  
868 Epidermis (RHE) test method. The assessment of skin irritation is positive with a score of RS1. The  
869 assessor conducts an expert review of the LLNA and suspects a false positive LLNA result. The 'Events in  
870 Rodent Lymphocytes' endpoint could be assigned as positive with low confidence; however, the  
871 negative *in silico* results are more reliable and relevant in this situation and the negative assessment  
872 carries over to the 'Events in Rodent Lymphocytes' endpoint with medium confidence. The GPMT data is  
873 negative with a reliability of RS1 since the study adhered to OECD 406 and the irritant effect was  
874 considered in the study design and interpretation of results. *In silico* models agree with the experimental  
875 GPMT result. The overall assessment of 'Skin sensitization in rodents' is negative with a high confidence,  
876 Figure 11b.

877 To further investigate the outcome in humans, the assessor conducted an *in silico* assessment using a  
878 set of alerts that were developed using HMT and HRIPT data as a reference database and no alerting  
879 structure were found. No human study data were available; however, DPT data were available and  
880 consecutive patients showed frequencies of 0% in a study. The absence of positive DPT results are  
881 indicative of no sensitization in humans, although a conclusion cannot be made from DPT data alone.

882  
883 Given the weight of evidence presented in Case 1a and 1b a final determination of the 'skin sensitization  
884 in humans' can be made. In this case, a well conducted GPMT carried significant weight towards the  
885 negative sensitization assessment with high confidence; reflecting the high reliability and relevance of  
886 the information. Other evidence supporting a negative assessment included a negative protein binding  
887 test which was reinforced by negative *in silico* models predictions of protein binding; and negative  
888 (Q)SARs predicting the 'Events in dendritic cells', and LLNA. The conflicting piece of information  
889 presented by the LLNA study was viewed as less reliable and relevant information due primarily to  
890 confounding irritant effects in the study. A second piece of conflicting information was presented by the  
891 KeratinoSens<sup>TM</sup> experimental study. While no specific explanation for this false positive was

892 determined, the body of negative evidence for the 'skin sensitization *in vitro*' endpoint supports the  
893 negative assessment and the low confidence reflects any uncertainty in the assessment of that  
894 endpoint. However, the 'sensitization *in vitro*' assessment does not discredit the 'skin sensitization in  
895 rodents' assessment. Since the *in vitro* and rodent endpoints are both equally relevant when the *in vitro*  
896 endpoint is derived through a defined approach, the endpoint that contains more reliable information  
897 contributes more to the overall confidence. The *in vitro* endpoint does not introduce any uncertainty in  
898 the GPMT experimental findings, and taken together with the DPT data, the final confidence score is  
899 high in this negative case, Figure 11c. There may be instances where a higher level of conservatism is  
900 necessary than presented. In such instances, the confidence score could be reduced to medium,  
901 although a change in the assessment might be difficult to justify.

#### 902 **4.3 Case 2a: Pro/pre-hapten assessment**

903 Figure 12a details the assessment for the mechanisms/effects that were considered in the case. A  
904 chemical is being screened for possible use in the cosmetics industry. It is expected to undergo  
905 metabolic transformation leading to the formation of quinones, which have a high probability to react  
906 via Michael addition (MA). There are positive alerts for dermal metabolism and the site of metabolism  
907 coincides with a pro-MA reactivity alert. Negative DPRA data are available and the DPRA study is  
908 assigned a reliability score of 1 based on adherence to OECD TG 442C. However, based on knowledge  
909 that the compound contains a pro-reactive feature that coincides with a site of metabolism, the  
910 relevance of the DPRA for testing the compound is challenged since any activity that results from a  
911 metabolic transformation may be missed. The DPRA test is considered not relevant for the compound  
912 tested, and the assessment of the 'Covalent interaction with skin proteins' is based on the assignment of  
913 a pro-reactive domain. Although there may be cases where a pro-reactive domain assignment does not  
914 lead to protein interaction due to deactivating features, a conservative approach to assessing the  
915 endpoint given a pro-reactive feature is to assign a positive assessment with a lowered confidence. No  
916 other *in vitro* data are available for the compound. A (Q)SAR was developed based on proprietary data  
917 for the KeratinoSens<sup>TM</sup> assay. The test compound is assessed as positive in the (Q)SAR, with the pro-MA  
918 feature identified as significant by the model. After a review of the (Q)SAR prediction, the 'Events in  
919 Keratinocytes' is assessed as positive with medium confidence. No data or models were available for the  
920 'Events in dendritic cells' endpoint. Given the positive assessment for 'Covalent interaction with skin  
921 proteins' and the 'Events in Keratinocytes', the overall assessment for the 'Skin Sensitization *in vitro*'  
922 endpoint is made using the "2 out of 3" approach. The overall assessment of the 'Skin Sensitization *in*  
923 *vitro*' endpoint is positive with low confidence based on the two aligned positive assessments and the

924 lower confidence score propagating to the endpoint, Figure 12a. It is possible to extrapolate the existing  
925 hazard information to the 'Skin sensitization in humans' endpoint and assess it as positive with low  
926 confidence.

#### 927 **4.4 Case 2b: Pro/pre-hapten assessment Example 2**

928 Consider an extension of the case presented in Section 6.3. LLNA data are not available for the test  
929 compound but are available for close analogs. In addition there is a low quality guinea pig test for the  
930 test compound that indicates a positive sensitization response. Read-across is performed using the  
931 LLNA data for the analogs. The analogs all contained the pro-reactive feature and formed a congeneric  
932 series that allowed interpolation of the LLNA EC3 value. The EC3 value was predicted to be 3.2%,  
933 indicative of a moderate sensitizer. The 'Events in rodent lymphocytes' endpoint was assessed as  
934 positive with medium confidence based on the read-across result. The guinea pig test is assigned a  
935 reliability score of RS5 based on deviations from OECD 406. A review of the study showed that for an  
936 induction concentration of 1%, the sensitization incidence is 100% suggesting that the compound could  
937 be classified as a Category 1A sensitizer. After an expert review of the study, the reliability score is  
938 increased to RS3. The overall assessment of the 'Skin Sensitization in Rodents' endpoint is assessed as  
939 positive, with medium confidence based on the weight of evidence presented by the LLNA read-across  
940 and guinea pig study.

941 The 'Skin sensitization in rodents' and the 'Skin sensitization *in vitro*' endpoints both support that  
942 assignment of a positive hazard for the 'skin sensitization in humans' with medium confidence. Figure  
943 12b shows the flow of information within the hazard assessment framework.

#### 944 **5. Reporting**

945 An important consideration towards *in silico* standardization, reproducibility and transparency is a  
946 consistent reporting format (Myatt et al. 2018). The general protocol (Myatt et al. 2018) describes a  
947 proposed reporting format that includes the elements that provide completeness of information. The  
948 report format is reproduced in Table 4 with a minor modification for the skin sensitization endpoint. In  
949 addition to the description of models, databases, and tools that were used, it is also recommended to  
950 describe any IATAs, DIPs or DAs that were used in deriving the overall assessment. The details that are  
951 suggested should allow another expert to repeat the process and achieve the same results. Further, the  
952 standardized report enables streamlined and consistent review of regulatory submissions across  
953 industries and endpoints. Section 5 of the Supplementary Material (SM5) provides an example of a  
954 report for sensitization hazard.

## 955 **6. Conclusion**

956 The skin sensitization *in silico* protocol presented here is the first publication to outline a systematic  
957 assessment of skin sensitization based on both experimental data and *in silico* predictions. It includes a  
958 HAF and provides general rules for the *in silico* toxicological assessment of chemicals within the  
959 framework. The framework is transparent and flexible as it does not require the generation of all  
960 endpoints to derive an overall assessment of 'skin sensitization in humans' and can accommodate  
961 quantitative and qualitative predictions and/or experimental results. There are cases where  
962 extrapolation to the human endpoint is possible and this has been described. The corresponding  
963 assessment of the confidence for all endpoints allows the protocol to be used in a variety of use cases.  
964 For example, assessments with low confidence scores may still have practical usage in screening or  
965 prioritization use cases. In addition, the protocol highlights experimental approaches or *in silico* models  
966 that could be incorporated into the HAF in the near future. Expert review is a critical element in any such  
967 procedure and items to consider as part of this review are listed to support a more consistent  
968 assessment. The standardization of the HAF for performing *in silico* methods is designed to support  
969 increased use and acceptance of *in silico* tools among regulatory agencies and industries alike.

## 970 **7. Acknowledgements**

971 Research reported in this publication was supported by the National Institute of Environmental Health  
972 Sciences of the National Institutes of Health under Award Number R43ES026909. The content is solely  
973 the responsibility of the authors and does not necessarily represent the official views of the National  
974 Institutes of Health.

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984 **Tables**985 Table 1. Sources of data for the development of *in silico* methods986 Table 2. Examples of deriving an overall assessment and confidence for the “skin sensitization *in vitro*”  
987 endpoint using the “AOP 2 out of 3” approach

988

989 Table 3: Factors increasing and decreasing confidence in a human study (Schulz, Altman, and Moher  
990 2010; Sibbald and Roland 1998)991 Table 4: Elements of an *in silico* toxicology report992 **Figures**993 Figure 1. A generic hazard assessment framework that shows the relationship between the key  
994 components of the protocol995 Figure 2. The hazard assessment framework describing the *in silico* components relevant for skin  
996 sensitization. *In silico* models could be developed for any effect or mechanism within grey boxes.

997

998 Figure 3. Adverse Outcome Pathway (AOP) for skin sensitization. MIE- molecular initiating event, KE (1-4)  
999 - Key Events 1-4.1000 Figure 4. The hazard assessment framework annotated with sections that discuss the assessment and  
1001 confidence score of each endpoint.

1002

1003 Figure 5. Decision tree showing how an overall assessment and confidence score could be derived for  
1004 the covalent interaction of skin proteins. The confidence scores are based on RS1 experimental data:  
1005 assuming relevant data and high reliability, and, in practice, confidence scores may need to be adjusted  
1006 based on reliability scores, SM Table 8. \*If a pro-reactivity domain is assigned and the metabolic site  
1007 (determined using structural alerts for skin metabolism) coincides with the pro-reactivity domain center  
1008 then the reversal in assessment occurs. If the metabolic site and the reactivity domain center do not  
1009 align then the assessment is inconclusive. <sup>§</sup>The inconclusive result is applicable in situations where  
1010 structural alerts could be used to determine if a structure is expected to undergo metabolism but not  
1011 identify the metabolites. In this case, since the reactivity of the metabolite cannot be confirmed, a  
1012 conclusion cannot be made on the assessment. If the reactivity of the metabolites could be predicted  
1013 then the final assessment depends on the metabolite reactivity.

1014 Figures 6A. Decision trees showing how an overall assessment and confidence score could be derived for  
1015 the 'events in keratinocytes'. The confidence scores here are based on RS1 experimental data: assuming  
1016 relevant data and high reliability, and, in practice, confidence scores may need to be adjusted based on  
1017 reliability scores.

1018

1019 Figures 6B. Decision trees showing how an overall assessment and confidence score could be derived for  
1020 the 'events in keratinocytes'. The confidence scores here are based on RS1 experimental data: assuming  
1021 relevant data and high reliability, and, in practice, confidence scores may need to be adjusted based on  
1022 reliability scores.

1023

1024 Figure 7. Decision tree showing how an overall assessment and confidence score could be derived for  
1025 the 'events in dendritic cells' based on the h-CLAT assay. The confidence scores here are based on RS1  
1026 experimental data: assuming relevant data and high reliability, and, in practice, confidence scores may  
1027 need to be adjusted based on reliability scores.

1028

1029 Figure 8. Decision tree showing how an overall assessment and confidence score could be derived for  
1030 the 'events in dendritic cells' based on the U-SENS™ and IL-8 Luc assay data. The confidence scores here  
1031 are based on RS1 experimental data: assuming relevant data and high reliability, and, in practice,  
1032 confidence scores may need to be adjusted based on reliability scores, SM Table 10.

1033

1034 Figure 9. Decision tree showing how an overall assessment and confidence score could be derived for the "Events  
1035 in rodent lymphocytes" based on the LLNA. The confidence scores here are based on RS1/2 experimental data  
1036 (except in the case of \*): assuming relevant data and high reliability, and, in practice, confidence scores may need  
1037 to be adjusted based on reliability scores. \*Concentrations tested in the LLNA are either non-irritating or mildly  
1038 irritating. The low confidence score reflects the non-specific increase in lymphocyte proliferation that could occur  
1039 with irritants.

1040 Figure 10. Decision tree showing how an overall assessment and confidence score could be derived for  
1041 the 'skin sensitization in rodents' endpoint based on guinea pig tests. The confidence scores here are  
1042 based on RS1 experimental data (except in the case of \*): assuming relevant data and high reliability,  
1043 and, in practice, confidence scores may need to be adjusted based on reliability scores. \*GPMT/BT

1044 challenge concentrations are non-irritating; however, deviations from OECD 406 may reduce the  
1045 relevance of the study and decrease the confidence in the endpoint.

1046

1047 Figure 11a. Derivation of the 'skin sensitization *in vitro*' endpoint using the "AOP 2 out of 3" approach  
1048 (Case 1a)

1049 Figure 11b. Derivation of the 'Skin Sensitization in Rodents' endpoint

1050 Figure 11c. Derivation of the 'skin Sensitization in Humans' endpoint from the weight of evidence  
1051 presented from the 'Skin Sensitization skin *in vitro*' and 'Skin Sensitization in Rodents' endpoints. DPT  
1052 data is also used to support the overall assessment.

1053 Figure 12a. Derivation of the 'skin sensitization *in vitro*' endpoint using the "AOP 2 out of 3" approach  
1054 (Case 2)

1055 Figure 12b. Derivation of the 'Skin Sensitization in Humans' using the "AOP 2 out of 3" approach (Case 2)

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- 1230

Journal Pre-proof

**Tables**Table 1. Sources of data for the development of *in silico* methods

<b>Database</b>	<b>Description</b>
<b>NTP-ICE</b>	Integrated Chemical Environment (ICE), an open access database with results from NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
<b>SkinSensDB</b>	SkinSensDB is a collection of data from published literature to facilitate the development of AOP-based computational prediction methods(Wang et al. 2017)
<b>ECHA-CHEM</b>	European Chemicals Agency (ECHA) database is an open access database containing data for chemicals manufactured and imported in Europe. Although the summaries are publicly available, extracting data in large amounts requires special consideration as the studies are proprietary
<b>TOXNET-HSDB</b>	Hazardous Substances Data Bank (HSDB) is an open source database that provides information on human exposure to potentially hazardous chemicals
<b>EURL-ECVAM-DB-ALM</b>	The European Union Reference Laboratory for alternatives to animal testing database service on alternative methods to animal experimentation is an open access database, containing information on percutaneous absorption
<b>CosIng</b>	European Commission database of current and historical data for cosmetic substances and ingredients
<b>RIFM</b>	The Research Institute For Fragrance Materials (RIFM) monographs contain human health and toxicological data for fragrance and flavor raw materials.
<b>Proprietary</b>	Databases generated within a specific institution. Structure activity relationship (SAR) fingerprints
<b>Literature</b>	Manual curation of peer-reviewed articles and published training sets such as (MTD Cronin, 1994)

Table 2. Examples of deriving an overall assessment and confidence for the “skin sensitization *in vitro*” endpoint using the “AOP 2 out of 3” approach

Assessment and confidence scores			Overall Assessment	Explanation
Covalent Interaction with Skin Proteins	Events in Keratinocytes	Events in Dendritic cells	Skin sensitization <i>in vitro</i>	
Positive, high confidence	Positive, high confidence	Negative, low confidence	Positive, high confidence	Positive, high confidence is the majority assessment
Negative, high confidence	Negative, high confidence	Positive, high confidence	Negative, medium confidence	Negative is the majority assessment, the confidence score is lowered based on a consideration of a third high confidence result
Positive, high confidence	Positive, medium confidence	Positive, low confidence	Positive, medium confidence	Positive is the majority assessment, the confidence score is medium based on three aligned calls with different confidence scores
Negative, high confidence	Negative, medium confidence	Positive, medium confidence	Negative, medium confidence	Negative is the majority assessment, the lower confidence score of the two aligned calls propagates to the overall assessment
Negative, Low confidence		Positive, Low confidence	Inconclusive	The assessments are non-concordant and the confidence scores are aligned

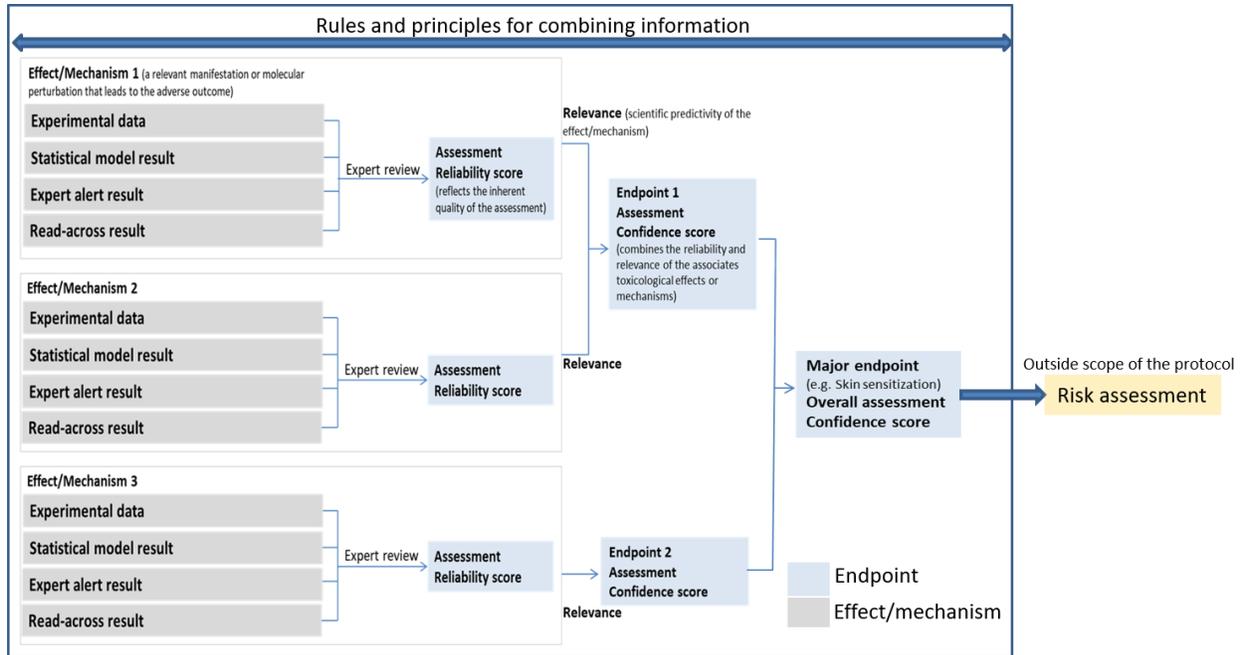
Table 3: Factors increasing and decreasing confidence in a human study (Schulz, Altman, and Moher 2010; Sibbald and Roland 1998)

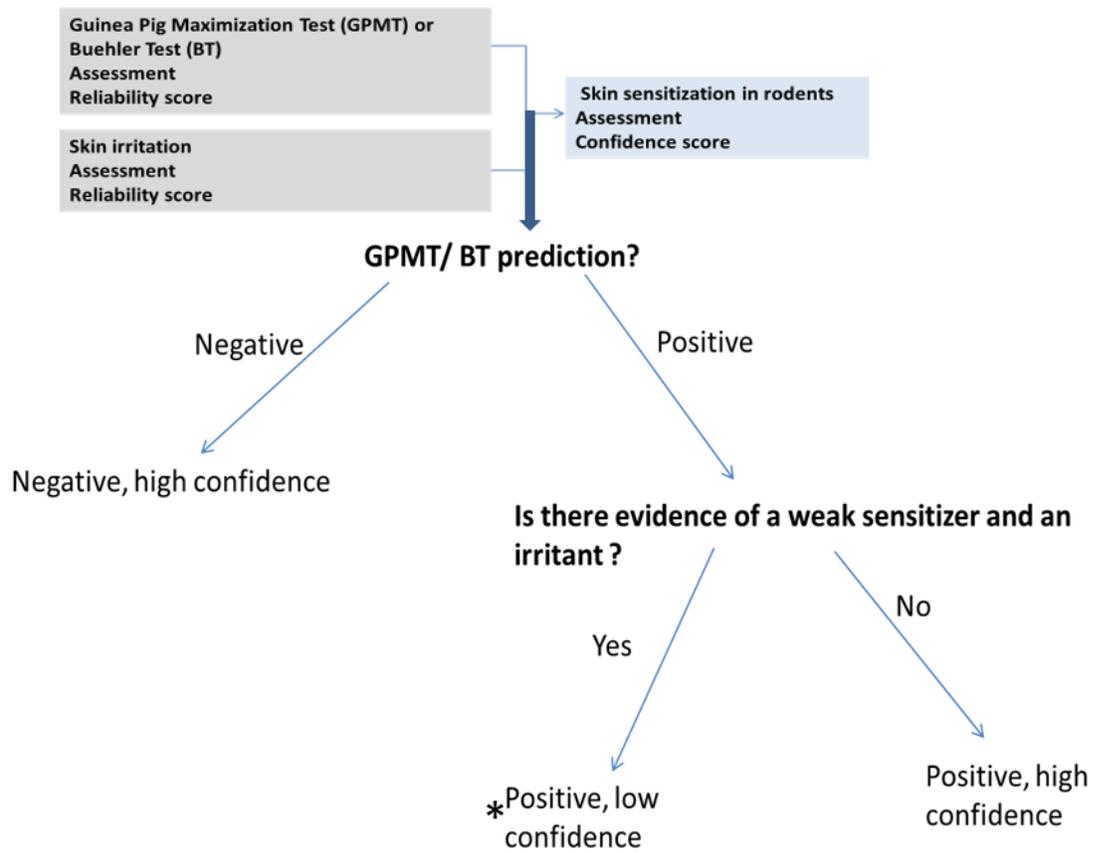
<b>Factors increasing confidence</b>	<b>Factors decreasing confidence</b>
Objective clearly stated and linked to measured outcome	Ambiguous objective, poorly linked to measured outcome
Randomized controlled study Randomized double-blind study	Uncontrolled and not randomized (or case report) No blinded control in study
Study conducted long enough to observe the effect	Study duration too short to observe the effect
Control substance application matches test substance application and represents the real-world exposure	Control substance application does not match test substance application or does not represent the real-world exposure scenario
Outcome clearly defined and measured through a quantitative endpoint	Subjective outcome based on perception
Statistical rationale behind determination of sample size	No rationale behind sample size selection
Description of study population available for review	No description of study population available

Table 4: Elements of an *in silico* toxicology report

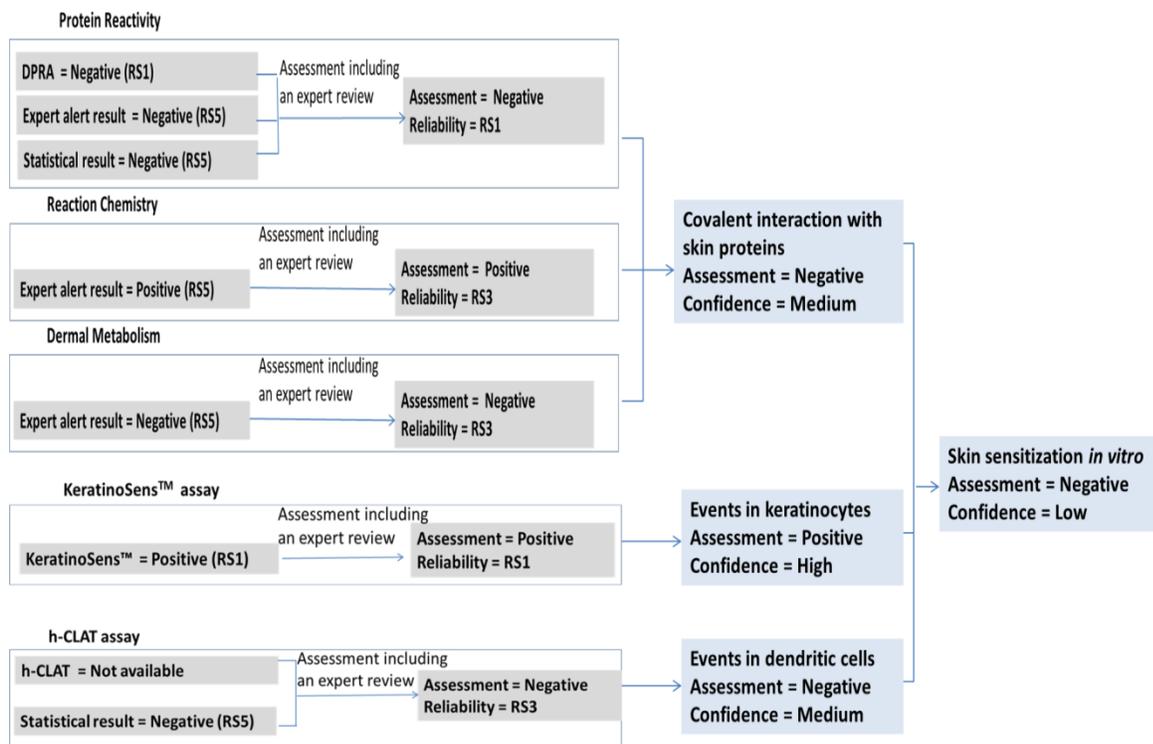
Section	Content
<b>Title page</b>	<ul style="list-style-type: none"> <li>- Title (including information on the decision context)</li> <li>- Who generated the report and from which organization</li> <li>- Who performed the <i>in silico</i> analysis and/or expert review, including their organization</li> <li>- Date when this analysis was performed</li> <li>- Who the analysis was conducted for</li> </ul>
<b>Executive summary</b>	<ul style="list-style-type: none"> <li>- Provide a summary of the study</li> <li>- Describe the toxicity or properties being predicted</li> <li>- Include a table or summary showing the following:               <ul style="list-style-type: none"> <li>o The chemical(s) analyzed</li> <li>o Summary of <i>in silico</i> results, reviewed experimental data and overall assessment for each toxicological effect or mechanism</li> <li>o Summary of toxicological endpoint assessment and confidence</li> <li>o Summary of supporting information</li> </ul> </li> </ul>
<b>Purpose</b>	<ul style="list-style-type: none"> <li>- Specification of the problem formulation</li> </ul>
<b>Materials and methods</b>	<ul style="list-style-type: none"> <li>- QSAR model(s), expert alerts, and other models used with version number(s) and any parameters set as part of the prediction (e.g., QMRF<sup>1</sup> format)</li> <li>- Databases searched with version number(s)</li> <li>- Description of any IATAs, DIPs, DAs used</li> <li>- Tools used as part of any read-across with version number(s)</li> </ul>
<b>Results of Analysis</b>	<ul style="list-style-type: none"> <li>- Details of the results and expert review of the <i>in silico</i> models and any experimental data, including results of the applicability domain analysis</li> <li>- Report of any read-across analysis, including source analogs and read-across justifications</li> </ul>
<b>Conclusion</b>	<ul style="list-style-type: none"> <li>- Summarize the overall analysis including experimental data, <i>in silico</i> methods and expert review</li> <li>- Final prediction that is based on expert judgment</li> </ul>
<b>References</b>	<ul style="list-style-type: none"> <li>- Complete bibliographic information or links to this information, including test guidelines referred to in the experimental data, etc.</li> </ul>
<b>Appendices (optional)</b>	<ul style="list-style-type: none"> <li>- Full (or summary) study reports used or links to the report, detailed (or summary) <i>in silico</i> reports, reports on the models used (e.g., QMRF reports)</li> </ul>

<sup>1</sup>QMRF – QSAR Model Reporting Format

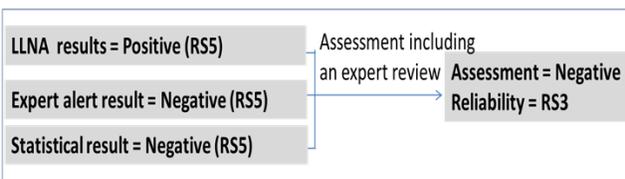




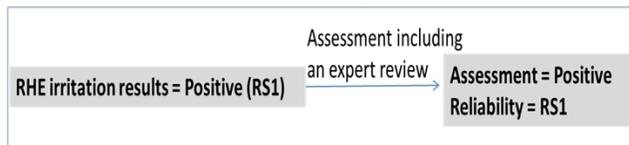
Journal



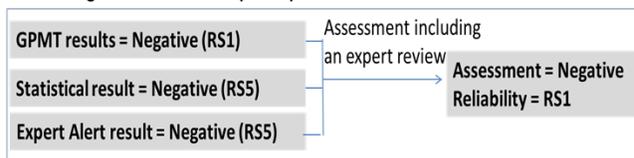
## Local Lymph Node Assay (LLNA)



## In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method



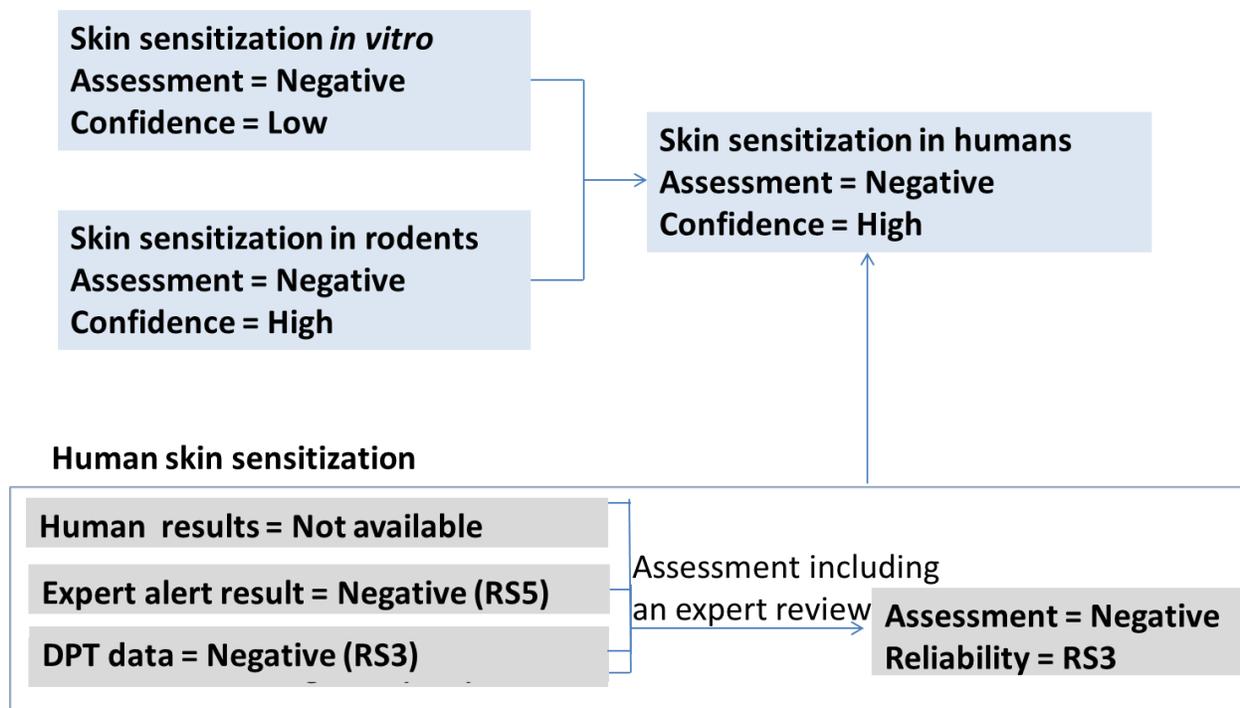
## Guinea Pig Maximization Test (GPMT)

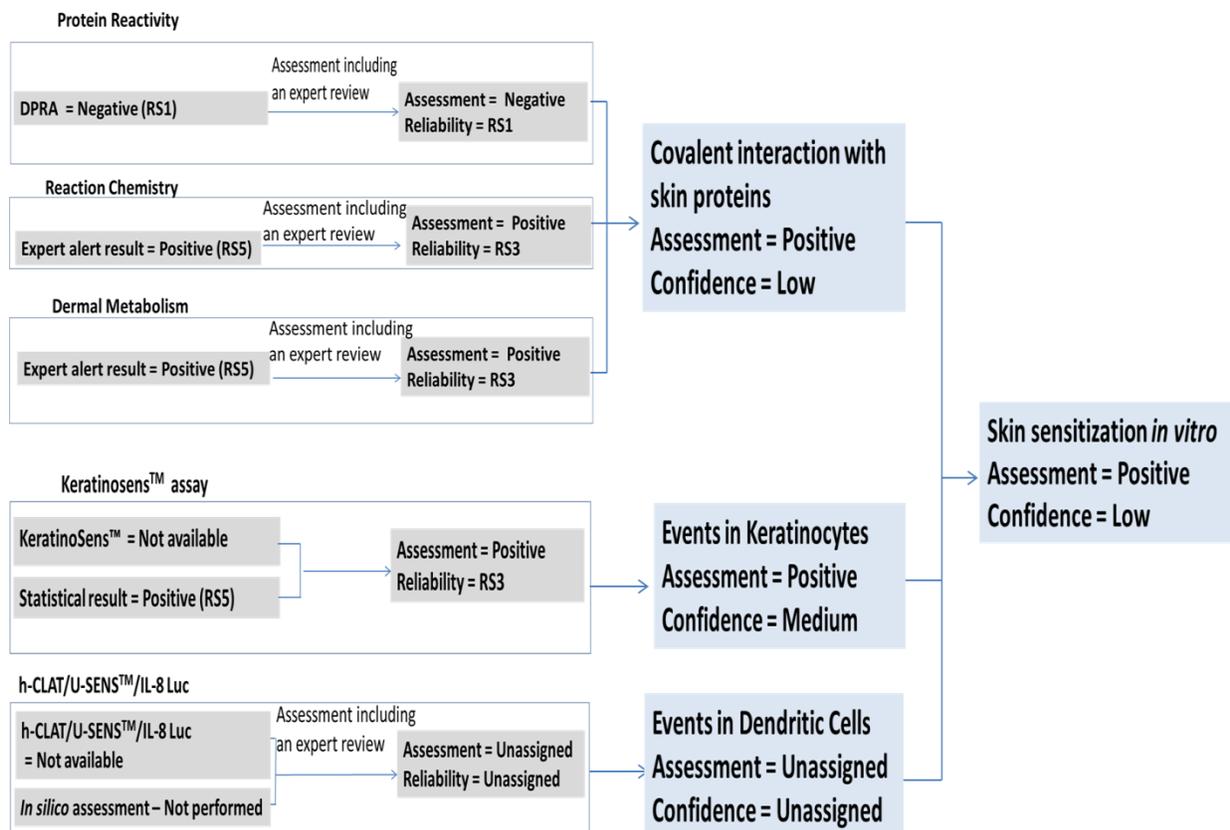


Events in rodent lymphocytes  
Assessment = Negative  
Confidence = Medium

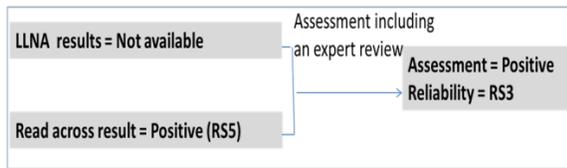
Skin sensitization in  
rodents  
Assessment = Negative  
Confidence = High

Journal Pre-proof



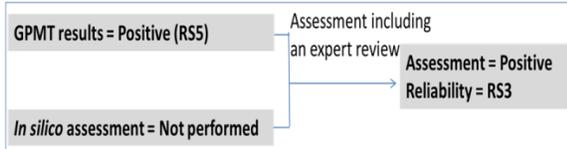


## Local Lymph Node Assay (LLNA)



Events in rodent lymphocytes  
Assessment = Positive  
Confidence = Medium

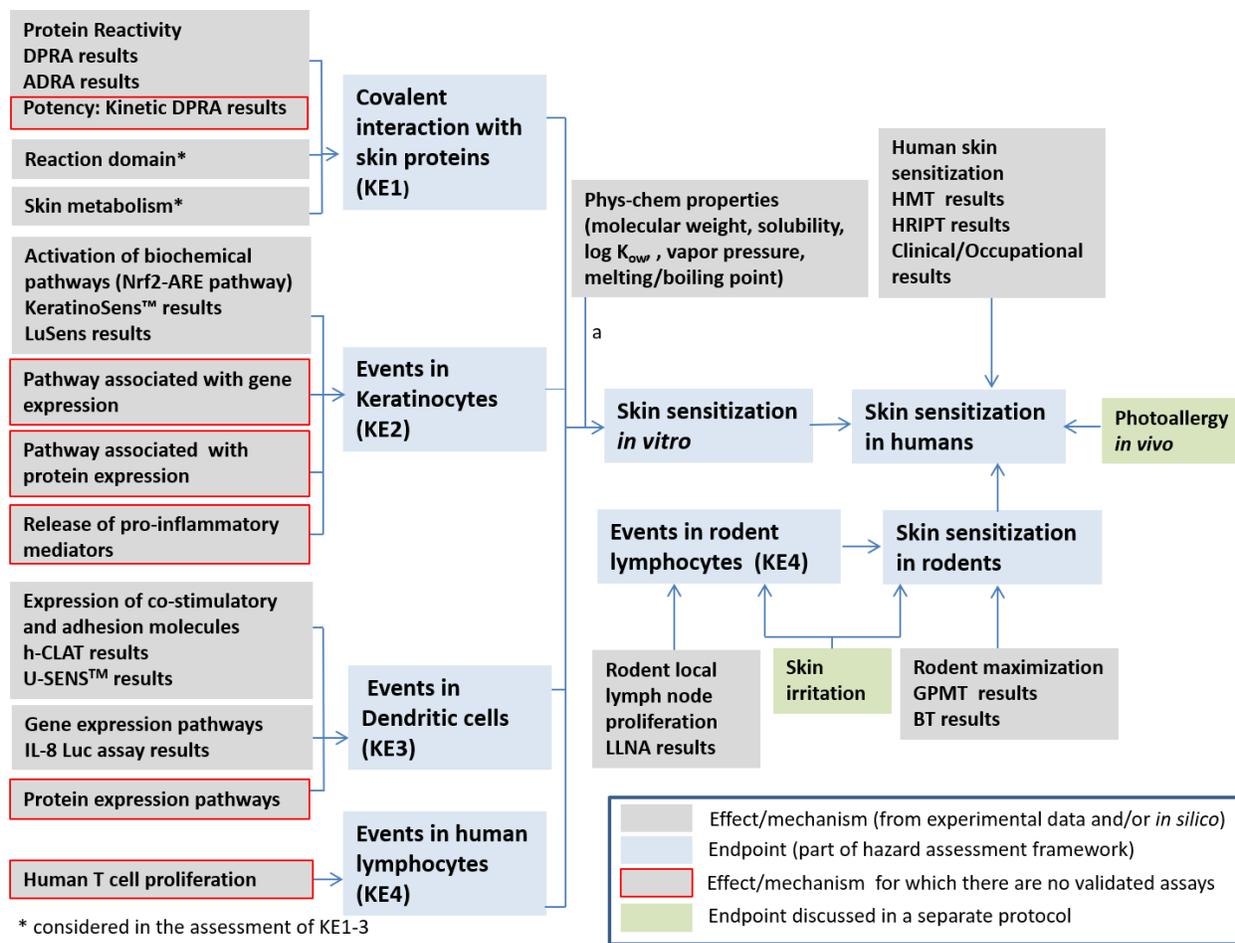
## Guinea Pig Maximization Test (GPMT)

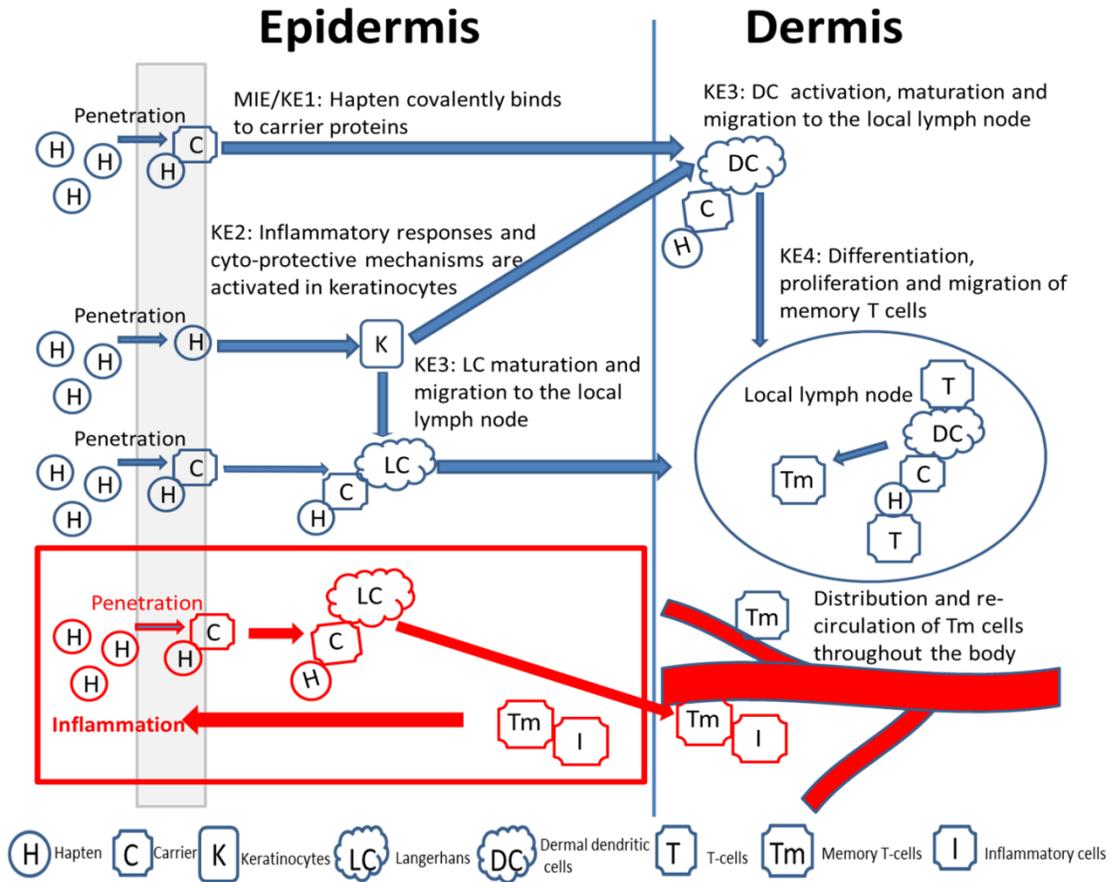


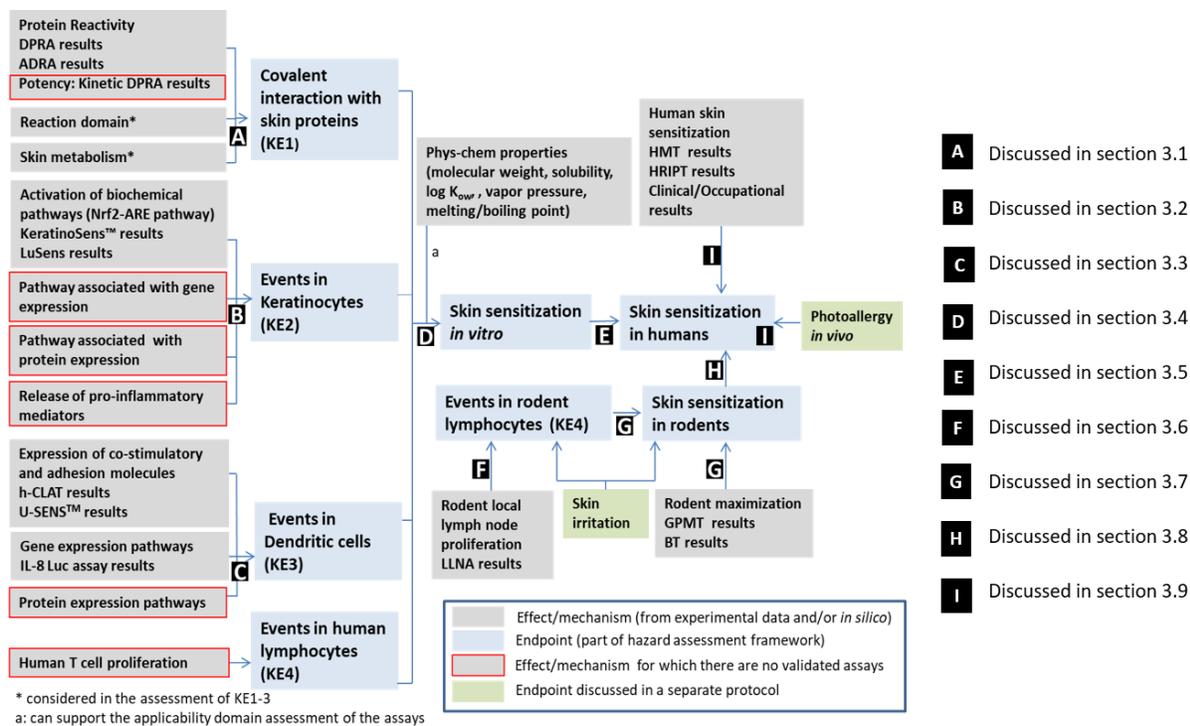
Skin sensitization in rodents  
Assessment = Positive  
Confidence = Medium

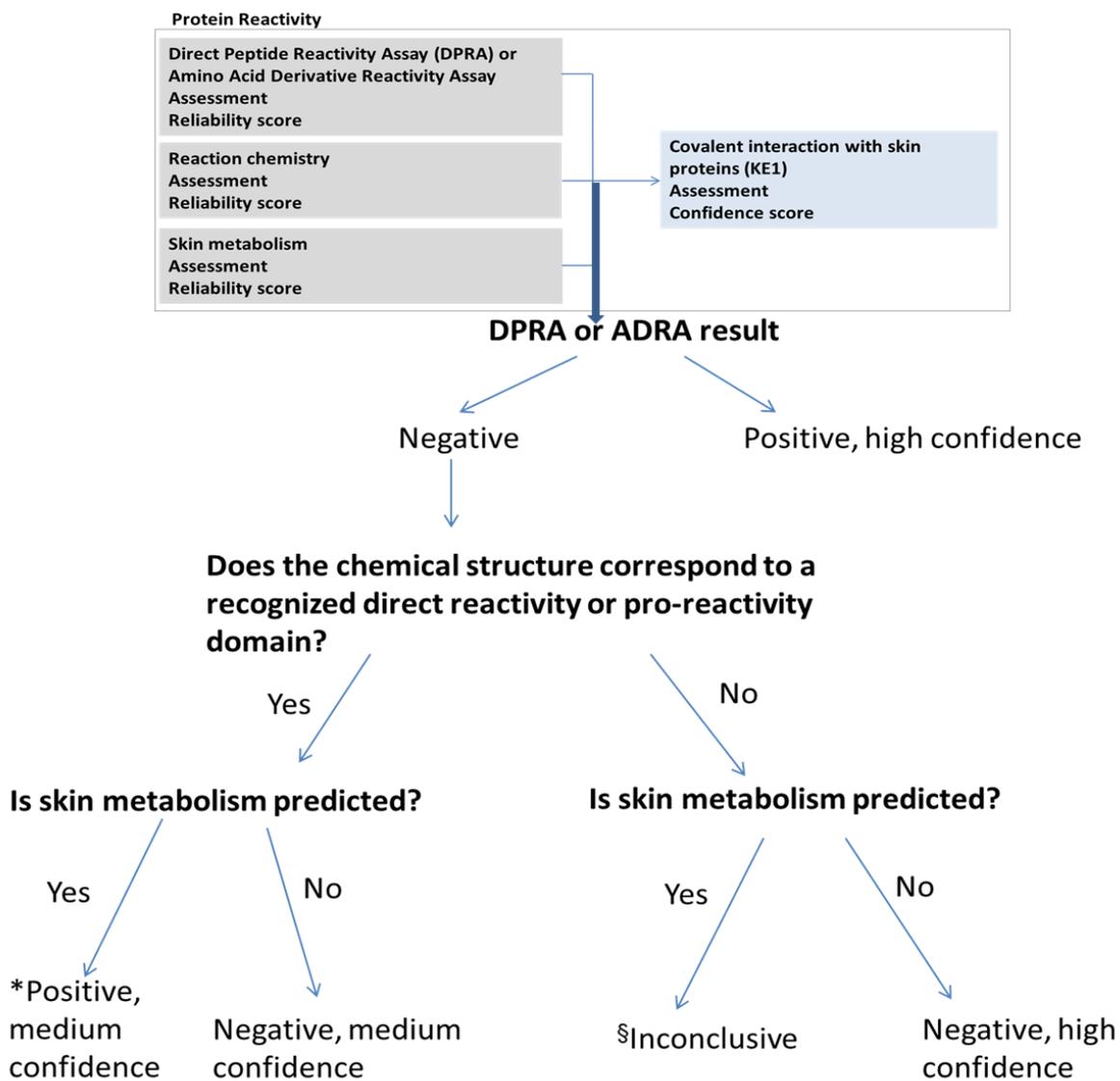
Skin sensitization in humans  
Assessment = Positive  
Confidence = Medium

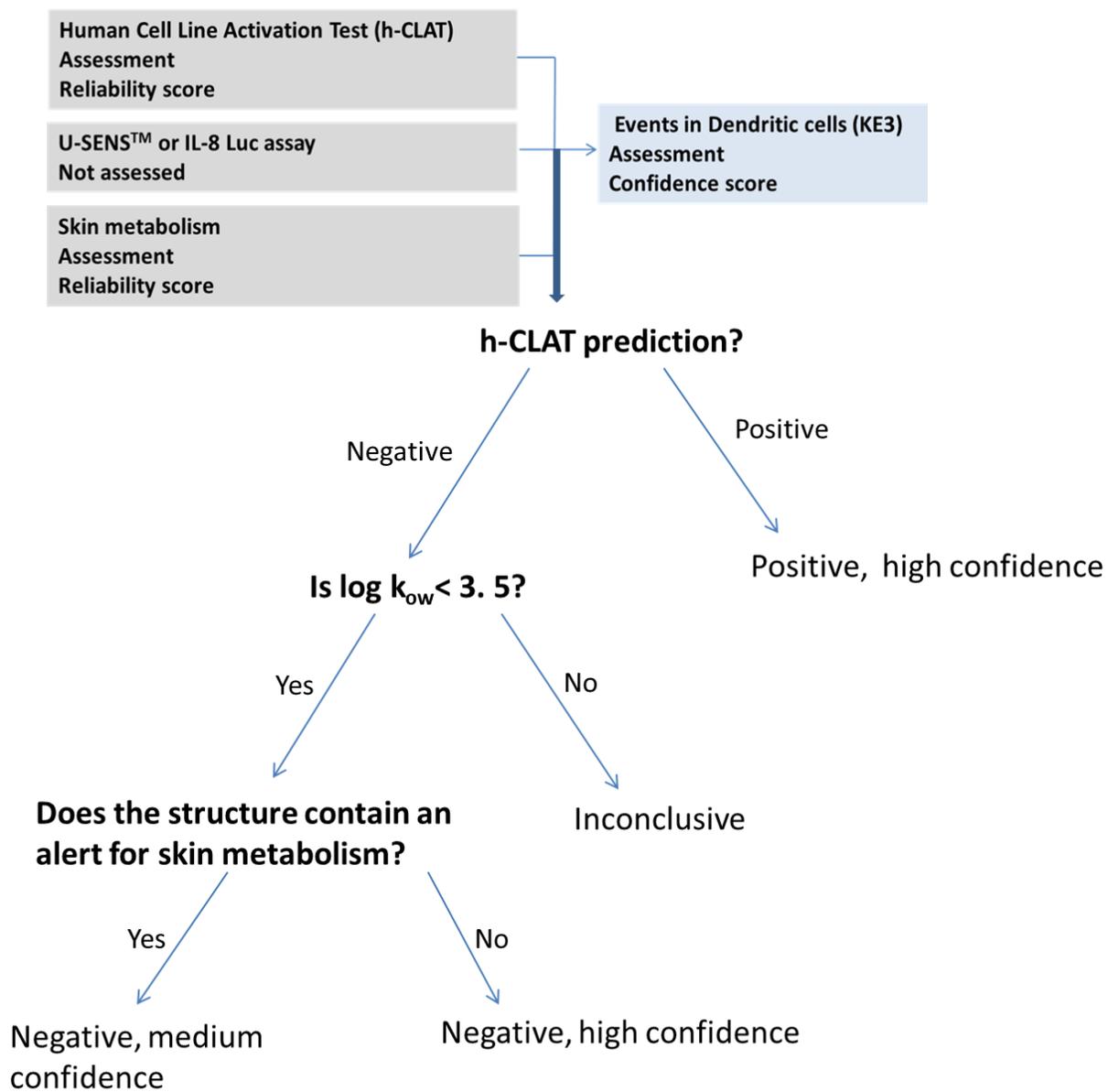
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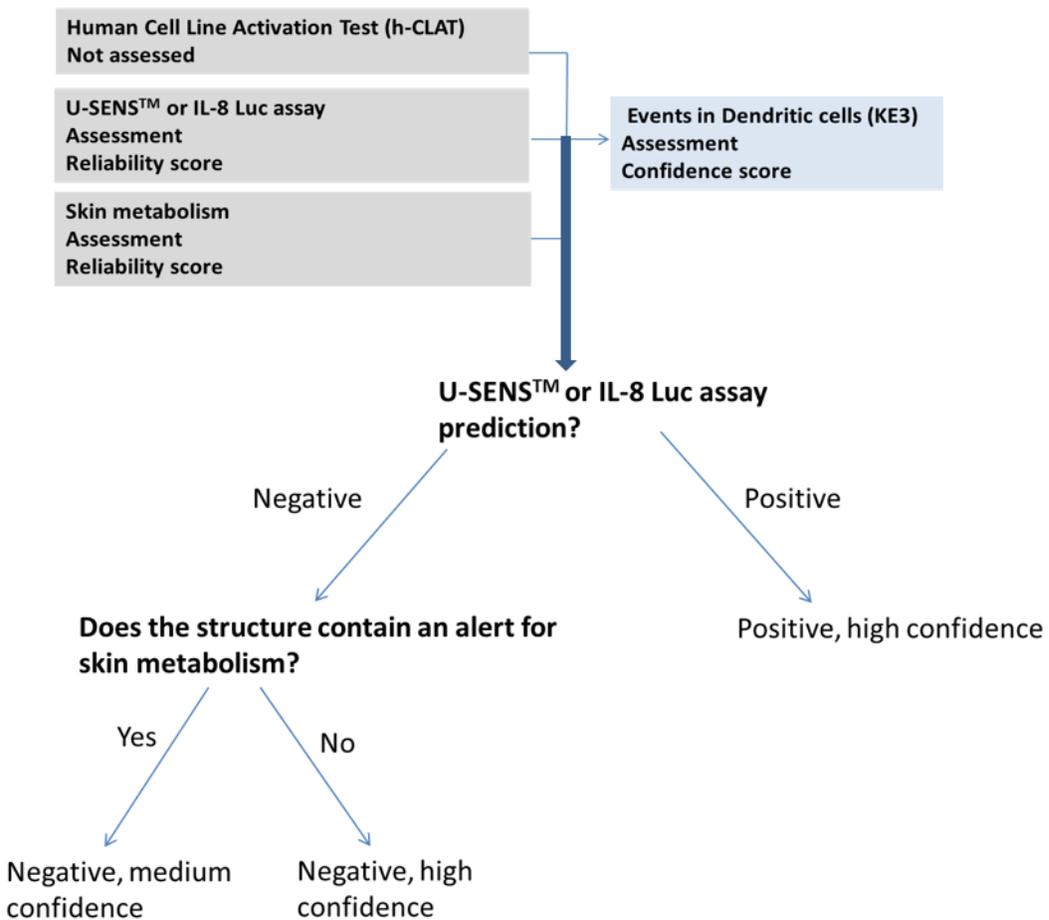


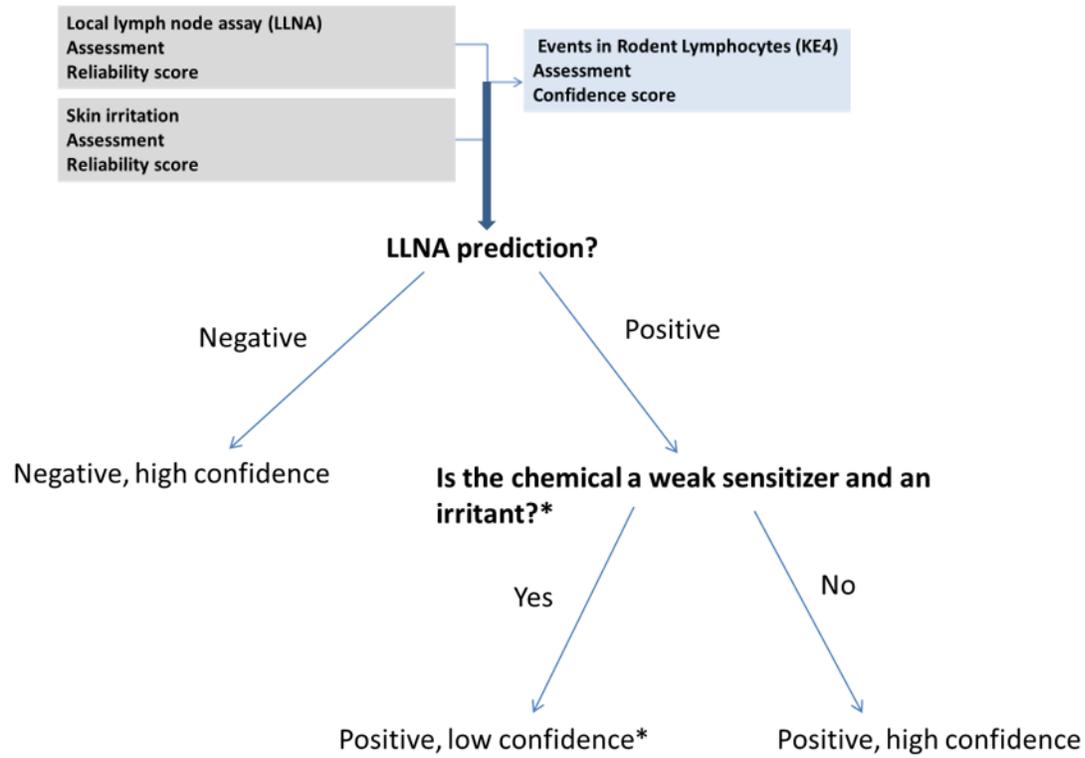


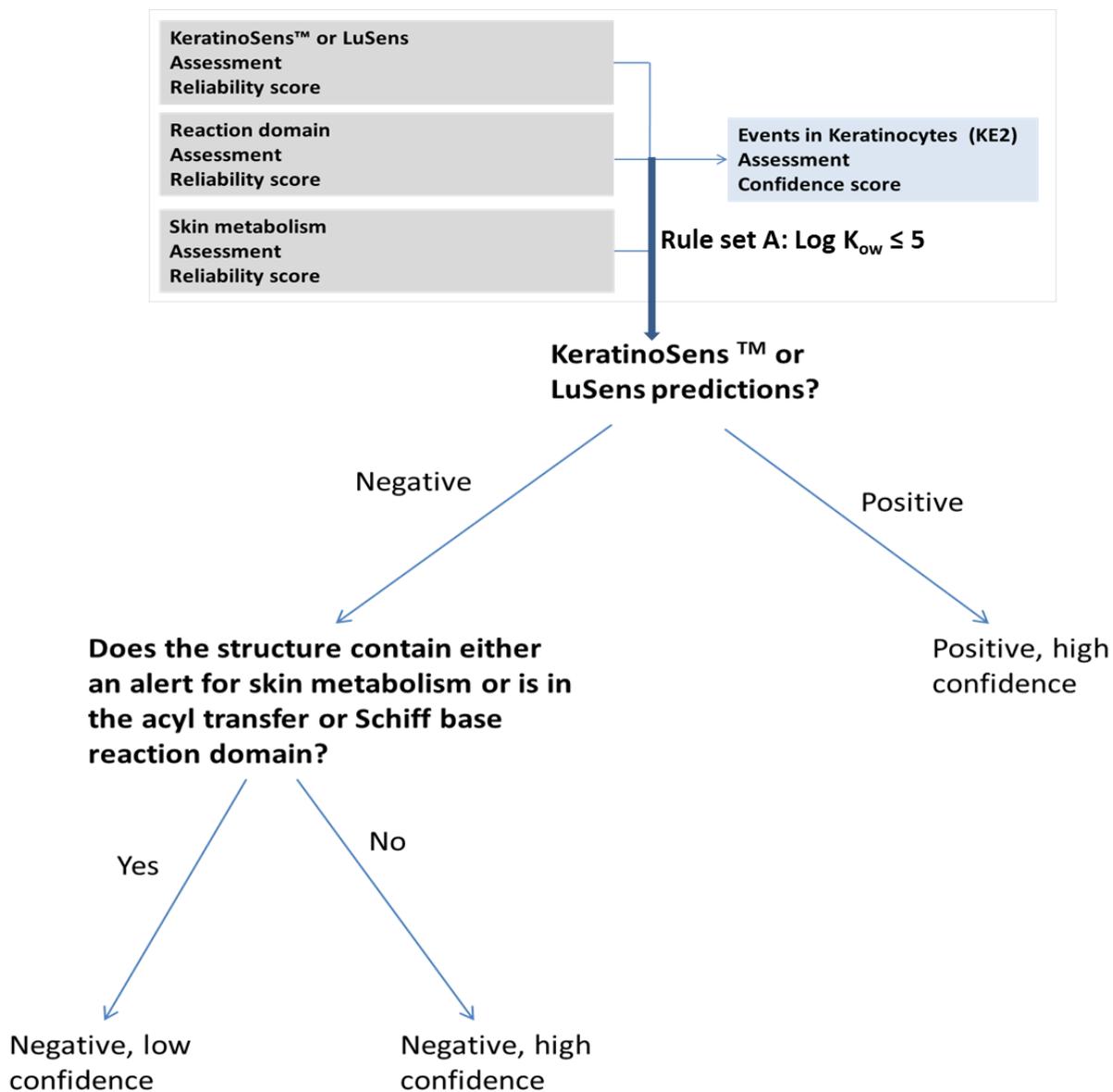


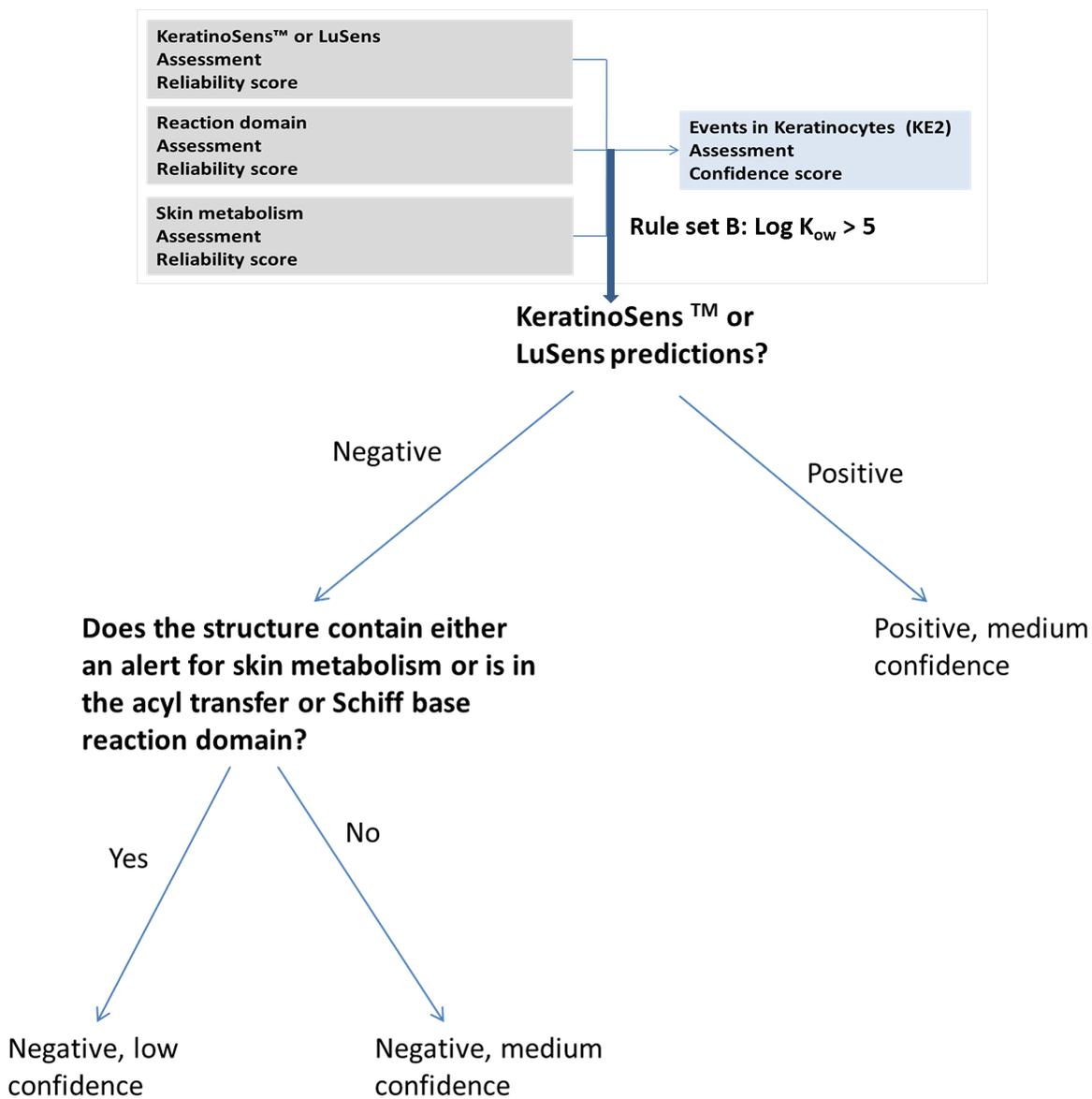












**Highlights**

1. Details a hazard assessment framework for skin sensitization that includes experimental data and *in silico* results
2. Defines rules and principles for deriving an assessment from the available information
3. Outlines criteria to be considered as part of an expert review of an assessment
4. A method for assigning confidence to skin sensitization assessments is proposed

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Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number R44ES026909. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: